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COMMENTARY

New Synthesis: Chemical Ecology and Sustainable Food Production

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Chemical ecology as an intrinsically multidisciplinary subject must be vastly expanded to provide new technologies for sustainable food production in the face of global population rise and climate change. No longer can we roll out the unsubstantiated claim that the science is in place and that all we need to do is to transfer the technology. Not even the rapid advances in molecular genetics and particularly the dramatically increased access to complete genomic sequences and associated informatics are sufficient. They would benefit greatly from the potentially holistic approach offered by chemical ecology. Annotation of genomes currently is the step limiting progress, and chemical ecology is ideally suited to assigning biological function to genes associated with biosynthesis of plant secondary metabolites (PSMs). I have been attempting to bring about a new public understanding of the need for further development of genetically modified organisms (GMOs) for food production, and have encountered from both the scientific community and the public media many naïve questions relating to what the questioner believes are issues that we have overlooked. Chemical ecology encompasses these issues of multitrophic interactions and unexpected impacts of highly active metabolites, as is exemplified in the rules for publication in this Journal.

This should not imply that all chemical ecologically based approaches are the most sustainable. However, Baulcombe, in the report that he led on producing for the senior scientific academy of the UK (Royal Society, 2009), outlined the technologies necessary for sustainable intensification of food production, and many of these objectives can be tackled by chemical ecology. Recently, Phalan et al. (2011) provided evidence that sparing is more viable in this way than sharing. Thus, it is towards intensification that chemical ecology should primarily be directed and not in trying to create systems where agricultural production shares the same land in an attempt to maintain or expand species diversity. Specifically, to reduce reliance on seasonal inputs such as fertilizer, seed, pesticides and the energy needed to deliver these, in addition to the cost of soil preparation, more effects must be delivered *via* the seed, and eventually there needs to be a large scale movement towards perennial crops, including replacement of the current arable staples. Crop losses to pests, diseases, and weeds harm the environment as well as farm profitability because the carbon footprint of bringing forth the crop, from which there would be loss, has already been created. Therefore, powerful, integrated new approaches to providing plant nutrients and pest control are needed. The tools for many of these approaches will be PSMs studied and developed in the context of chemical ecology. Development can be by breeding but with newly available advances, such as alien introgression, that enable introduction of more potent traits from ancestral plants. Probably the most powerful approach will be *via* GMOs. For example, exploitation of defense benzoxazinoids in cereals such as rye, maize, and wheat, mostly lost from modern varieties and hybrids during breeding, could be enhanced or reintroduced, potentially providing control against pests and probably disease and weeds. By introducing nitrogen fixation into a wider range of crops, or into their rhizospheres, we can expect to harness allelopathy to reduce losses of fixed nitrogen as the powerful noxious greenhouse gas, nitrous oxide. Animal husbandry will also benefit in terms of new PSM-based pest control and use of molecular markers for animal breeding. Those geographic regions that support only grasslands for meat and dairy production could be made more acceptable by regulating the production of methane, the other major agrogenic greenhouse gas, by allelopathic traits in the grassland flora. Potential disadvantages and non-target effects will be studied as part of the development process.

Management of gene expression can be achieved by naturally occurring elicitors such as the development of *cis*-jasmone. Opportunities will involve not just other elicitors that can naturally penetrate the plant cuticle and effect systemic upregulation of defense genes, but will give rise to a new way of activating useful genetic traits by the development of sentinel plants. Such sensitive plants detect problems, not just pests, diseases, and weed competition but also depleted or excess nutrients and water, and signal to the main crop of smart plants; the natural response to the signals comprising small lipophilic molecules linked to gene expression (by GM). Perennial plants, as pointed out in The Royal Society report (2009) will be the ultimate goal, and sentinel plants will be a means of managing the necessary genetics for plant traits without the need for passing through the crop with energy intensive implements.

Baulcombe called for vastly increased expenditure in research and in education. The various disciplines needed to provide the new science must be closely linked, and chemical ecology should provide the overall framework. Both high science and applied and agricultural work are necessary. Functional genes causing useful biological effects need to be identified and expression ensured by elicitors where necessary. Bioassay (i.e. phenotyping) guided fractionation, the underpinning approach of chemical ecology for identifying the new crop management tools, is too often ignored. Many studies merely involve trivial perturbation of natural physiology by randomly adding, often very weakly active, natural products in high physiological excess. We must stop doing this and not give plausibility to those that do. We must show the way through high quality science and thereby persuade the world of the contribution that chemical ecology can make to sustainable intensification of food production.

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The Key Role of 4-methyl-5-vinylthiazole in the Attraction of Scarab Beetle Pollinators: a Unique Olfactory Floral Signal Shared by Annonaceae and Araceae

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Abstract Cyclocephaline scarabs are specialised scent-driven pollinators, implicated with the reproductive success of several Neotropical plant taxa. Night-blooming flowers pollinated by these beetles are thermogenic and release intense fragrances synchronized to pollinator activity. However, data on floral scent composition within such mutualistic interactions are scarce, and the identity of behaviorally active compounds involved is largely unknown. We performed GC-MS analyses of floral scents of four species of *Annona* (magnoliids, Annonaceae) and *Caladium bicolor* (monocots, Araceae), and demonstrated the chemical basis for the attraction of their effective pollinators. 4-Methyl-5-vinylthiazole, a nitrogen and sulphur-containing heterocyclic compound previously unreported in flowers, was found as a prominent constituent in all studied species. Field biotests confirmed that it is highly attractive to both male and female beetles of three species of the genus *Cyclocephala*, pollinators of the studied plant taxa. The origin of 4-methyl-5-vinylthiazole in plants might be associated with the metabolism of thiamine (vitamin B1), and we hypothesize that the presence of this compound in unrelated lineages of angiosperms is either linked to selective expression of a plesiomorphic biosynthetic pathway or to parallel evolution.

Keywords Floral scent · Beetle pollination · Gas chromatography-mass spectrometry (GC-MS) · Olfactory-mediated attraction

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Introduction

For numerous species of extant angiosperms, the emission of floral volatiles plays a role in breeding success. Such species rely on chemically mediated strategies for attracting effective pollinators (Faegri and van der Pijl, 1979; Raguso 2001, 2008). It is generally agreed that the broad spectrum of known flower visitors is matched by an equally vast assortment of floral volatiles of diverse origins, with a myriad of possible fragrant combinations (Dobson, 2006; Knudsen et al., 2006).

This chemical diversity is a portrait of the evolutionary processes of diversification and speciation involved in scent-mediated pollination mutualisms (Dobson, 2006).

Among some highly specialised pollination systems, such as those involving deceptive *Ophrys* orchid flowers and male solitary bees (Schiestl et al., 1999), scent-driven behavioral reactions of pollinators are triggered by common volatile compounds that act synergistically according to specific relative ratios in the overall floral bouquets (Raguso, 2008). In other systems, however, it has been shown that occasionally uncommon single compounds induce taxa-restricted attractive response of pollinators (Dodson et al., 1969; Schiestl et al., 2003). In these cases, described as 'private communication channels', the specificity of the olfactory signal is ensured by rare compounds synthesized in unusual pathways, unlikely to be reproduced by chance (Raguso, 2008). As an example, Australian sexually deceptive *Chiloglottis* orchids attract their highly specific pollinators with one or few extremely uncommon volatiles, i.e., chiloglottones. Sympatric species typically emit different single chiloglottones or different blends of compounds that attract different pollinator species (Peakall et al., 2010). Potential 'private communication channels' also have been documented among highly specialized plant-pollinator interactions involving male euglossine bees (Apidae, Euglossini) (Whitten et al., 1986) and fig wasps (Agaonidae) (Chen et al., 2009). It is likely that such systems are more common than what is currently documented, but the scarce knowledge of floral scent chemistry is still a barrier in pollination studies (Knudsen, et al., 2006).

Cyclocephaline scarab beetles (Scarabaeidae, Cyclocephalini) are predominant among the highly specialized pollinators in the Neotropics, implicated with the reproductive success of an estimated 900 Neotropical species among the Annonaceae, Araceae, Arecaceae, Cyclanthaceae, Magnoliaceae and Nymphaeaceae (Beach, 1982; Gottsberger, 1986; Schatz, 1990; Ervik and Knudsen, 2003). As the result of convergent adaptive evolution, several lineages within these families exhibit shared features in floral morphology and physiology, configuring what is broadly acknowledged as 'cyclocephaline scarab pollination syndrome' (Gottsberger, 1990). The night-active scarabs are attracted to flowers or inflorescences by intense odors given off during episodes of floral thermogenesis (Gottsberger and Silberbauer-Gottsberger, 1991; Seymour et al., 2003). Warm shelter and nutritious flower tissues await the pollinators inside floral chambers, where they feed and mate (Gottsberger, 1990).

Even basic information about floral scent composition within cyclocephaline scarab beetle-pollinated taxa is meager (Dobson, 2006). Nonetheless, a few available studies portray obvious diversity; oxygenated terpenoids, esterified fatty acid derivatives, C5-branched chain compounds, and uncommon methoxylated and hydroxylated benzenoids have all been reported as major constituents in these angiosperms (Azuma et al., 1999; Ervik et al., 1999; Schultz et al., 1999; Ervik and Knudsen, 2003; Gibernau et al., 2003; Maia et al., 2010). Verified behavioral response, however, only has been described for methyl 2-methylbutyrate, the main component in flowers of *Magnolia ovata* (Magnoliaceae) pollinated by *Cyclocephala literata* (Gottsberger et al., 2012). Compounds mediating other interactions within these highly specialized pollination systems are unknown.

In the present study, gas chromatography—mass spectrometry (GC-MS) was used to investigate the floral scent composition of four species of Annonaceae (magnoliids; *Annona* spp.) and one Araceae (monocots; *Caladium bicolor*), all pollinated by cyclocephaline scarabs of the genus *Cyclocephala*. The behavioral activity of a prominent compound isolated in the analyzed samples was assessed in field bioassays. The main questions we posed were: a) how similar is the floral scent composition in these two phylogenetically distant groups of angiosperms (Annonaceae and Araceae) pollinated by cyclocephaline scarab beetles? b) is the most prominent compound in these floral fragrances sufficient for (specific) pollinator attraction?

Methods and Materials

Study Sites Both the collection of floral scent samples and behavioral tests were conducted in two localities in Brazil where native populations of the selected species of *Annona* and *Caladium bicolor* naturally occur (Fig. 1).

The first site corresponds to remnants of *cerrado sensu lato* covering an area of ca. 100 ha. inserted in agricultural land and pastures in the municipality of Botucatu, Central Brazil (22° 52' S, 48°26' W; altitude ca. 850 m). Local vegetation is a mixture of the central Brazilian *cerrado* (open tree and scrub woodland to low-tree and scrub savanna), with scattered patches of mesophytic, more or less evergreen Atlantic forests (Gottsberger and Silberbauer-Gottsberger, 2006). Local rainfall regime is semi-humid, and over 80 % of the average annual precipitation (ca. 1,300 mm) occurs during the warmer months of October through March. Mean monthly temperatures vary between 16 °C in July and 23 °C in February (CEPAGRI, 2012).

At this site, henceforth referred to as *Botucatu*, populations of *Annona crassiflora*, *A. coriacea*, and *A. dioica* grow sympatrically and exhibit staggered flowering peaks between the months of October and December. All three species are essentially pollinated by *Cyclocephala atricapilla*, with erratic visits from few other congenerics (Gottsberger, 1989).

The second site corresponds to a private reserve of Atlantic Forest, covering ca. 66 km^2 comprised of scattered fragments inserted in a sugarcane monoculture matrix, property of Usina São José S/A Sugarcane Company, municipality of Igarassu, north-eastern coast of Brazil (7°49' S, 35°02' W; altitude ca. 110 m). Local rainfall regime is perhumid, and over

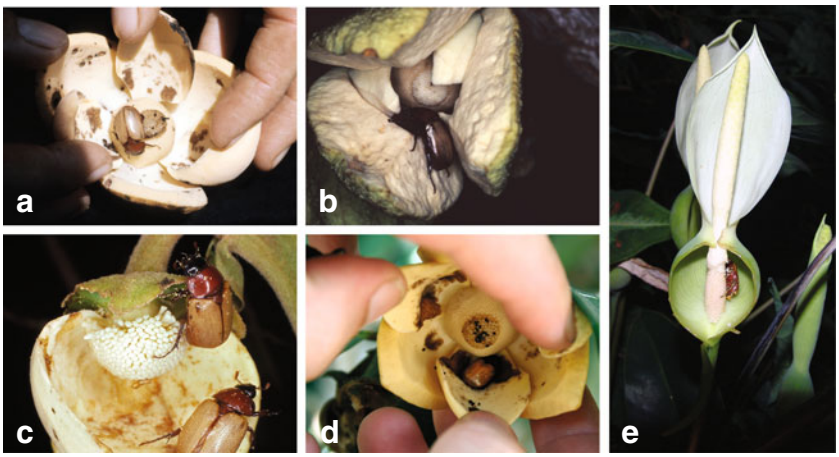

Fig. 1 Investigated plant species with *Cyclocephala* pollinators (Scarabaeidae, Dynastinae): **a** *Annona coriacea*, **b** *A. crassiflora*, **c** *A. dioica*, **d** *A. montana* (Annonaceae, magnoliids), **e** *Caladium bicolor* (Araceae, monocots)

80 % of the average annual precipitation (ca. 1,800 mm) occurs between February and August. Mean monthly temperatures vary between 26 °C in March and 23 °C in August (ITEP, 2012).

At this site, henceforth referred to as *Igarassu*, native *Caladium bicolor* populations bloom between March and May (Maia and Schlindwein, 2006). *Annona montana* populations flower year long, but are in full bloom between January and March. *Cyclocephala celata* is the exclusive local pollinator of *Caladium bicolor*. *Cyclocephala vestita* is the only known pollinator locally associated with *A. montana* (H. Teichert, unpublished data).

Volatile Collection and Chemical Analyses Floral scent samples were collected *in situ* using standard dynamic headspace extraction methods (Raguso and Pellmyr, 1998), during the flowering period of the selected species in 2008–2009. During the female phase of anthesis, flowers (*Annona* spp.) or inflorescences (*Caladium bicolor*) were individually enclosed in polyacetate bags. Scented air was drawn from the bags at a constant flow rate of 200 ml.min^{-1} for 30–120 min through glass cartridges filled with 25 mg adsorbent polymer, a 1:1 weight mix of Tenax and Carbotrap. After collection, the cartridges were eluted with 9: 1 hexane: acetone solvent and analyzed by combined gas chromatography-mass spectrometry (GC-MS) using methods detailed in Kaiser and Tollsten (1995) and Dötterl et al., (2006). To determine the total amount of scent in these samples, we added an internal standard (10 μg 3-chloro-4-methoxy toluene) prior to injection. Smaller cartridges filled with 3 mg of the adsorbent mix were used for sampling unbagged flowers of *Annona coriacea* and *A. crassiflora* (200 ml.min^{-1} flow, 2 min duration). These samples were thermally desorbed and analyzed by GC-MS, according to methods described by Dötterl et al., (2005). We calculated the total scent emission (absolute amount) of samples analyzed by thermal desorption by injecting known amounts of monoterpenoids, benzenoids, and fatty acid derivatives. The mean response of these compounds (mean peak area) was used to determine the total amount of scent available in the samples (Dötterl et al., 2006). Simultaneous collections of the surrounding air were performed for both sampling techniques to distinguish between floral/inflorescence compounds and ambient contaminants.

Behavioral Activity of 4-Methyl-5-vinylthiazole At *Botucatu*, biotests were conducted during November 2009. Undiluted 4-methyl-5-vinylthiazole (≥ 97 % purity, Sigma-Aldrich; 80 μl) was applied to pieces of white filter paper, cut to the approximate size of the perianth of *Annona* flowers. To determine the total amount of scent released by decoy flowers (N=5), we applied the same sampling method used for inflorescences of *Caladium bicolor* and flowers of *Annona dioica* and *A. montana* (see above). The total amount of 4-methyl-5-vinylthiazole emitted by the decoy flowers was calculated at 100 μg h^{-1}, similar to the values obtained from a single bagged flower of *Annona* under the same environmental conditions (Table 1). The decoys were fixed to branches of *Annona* trees or laid on the ground. On four different occasions, we installed sets of 2, 2, 4 and 5 decoys distant ca. 2 m from each other. At regular 10 min intervals, we collected the insects that settled over the decoys between 17:30–21:00 h, when cyclocephaline scarab beetles are most active. Baitless paper flowers were used as paired controls. Biotests at *Igarassu* took place between January and April 2010. We cut out pieces of dental cotton roll (2 cm sections) and pushed them into 4 ml clear glass vials (Supelco, Bellefonte, PA, USA), so that the tip of the cotton rolls leveled with the rim opening of the vials. Each dispenser was impregnated with 100 μl undiluted 4-methyl-5-vinylthiazole and applied to 'Japanese beetle' traps built based on a model by Trécé, USA (Bengtsson et al., 2009). Traps were hung chest-high from tree branches along borders of rainforest patches, 25 m apart from one another. Loaded dispensers (N=5) were bagged individually and sampled through dynamic headspace extraction, applying the same method used for the filter

Table 1 Relative amounts (mean±SD) of 4-methyl-5-vinylthiazole and other tentatively identified floral scent compounds (pooled according to compound class) in four species of *Annona* (Annonaceae, magnoliids) and *Caladium bicolor* (Araceae, monocots), all of which exclusively pollinated by cyclocephaline scarab beetles (Scarabaeidae, Cyclocephalini). Floral scent samples were obtained during the female phase of anthesis

Compound	*mw*	*A. cor.*	*A. cra.*	*A. dio.*	*A. mon.*	*C. bic.*
Total number of compounds		6	4	8	3	17
Number of analyzed samples		2	6	3	4	4
Average (min-max) total amount of scent per flower/inflorescence (μg/h)[a]		95 (61-130)	45 (tr-148)	9 (2-16)	90[b] (6-194)	2,178 (113-6,206)
Aliphatics						
3-Pentanyl acetate	130	–	–	–	–	0.81
Benzenoids and phenylpropanoids						
p-Methoxystyrene	134	–	–	0.71	–	–
Anisaldehyde	136	–	–	0.07	–	–
Veratrole	138	–	–	–	–	0.30
Methyl salicylate	152	–	–	–	–	15.56
Eugenol	164	–	–	0.22	–	–
Methyl 2-methoxybenzoate	166	–	–	–	–	0.43
1,2,4-Trimethoxybenzene	168	–	–	–	–	0.01
1,3,5-Trimethoxybenzene	168	–	–	–	–	31.49
1,2,3,5-Tetramethoxybenzene	198	–	–	–	–	0.01
Nitr. and/or sulp. cont. compounds						
Indole	117	–	–	0.05	–	0.01
4-Methyl-5-vinylthiazole	125	97.49	97.95	98.36	99.63	36.25
4-Methyl-5-formylthiazole	127	0.22	1.58	0.24	0.05	0.03
Terpenoids						
α-Pinene	136	–	0.43	–	–	–
β-Pinene	136	–	0.04	–	–	–
β-Myrcene	136	–	–	–	–	0.01
(*E*)-β-Ocimene	136	–	–	–	0.31	0.01
Linalool	154	–	–	–	–	8.73
Dihydro-β-Ionone	194	2.26	–	–	–	0.07
β-Caryophyllene	204	–	–	0.32	–	–
α-Humulene	204	–	–	0.02	–	–
Unid. *m/z*: 119,91,121,43,105,134		0.01	–	–	–	–
Unid. *m/z*: 121,107,91,93,136,192		0.01	–	–	–	–
Miscellaneous cyclic compounds						
(*E*)-Jasmone	150	–	–	–	–	0.05
(*Z*)-Jasmone	150	–	–	–	–	6.22
Methyl jasmonate	210	0.01	–	–	–	0.02
Fatty acid derivatives		–	–	–	–	0.81
Benzenoids and phenylpropanoids		–	–	1.00	–	47.79
Nitr. and/or sulp. cont. compounds		97.71	99.53	98.65	99.68	36.29
Terpenoids		2.28	0.47	0.34	0.31	8.82
Miscellaneous cyclic compounds		0.01	–	–	–	6.29

A. cor. Annona coriacea, *A. cra.* A. crassiflora, *A. dio.* A. dioica, *A. mon.* A. montana, *C. bic.* Caladium bicolor

[a] Scent amount trapped cannot be compared among species as different methods were used for scent collection (e.g. ,bagged vs. unbagged, see Methods and Materials)

[b] total amount was determined for three aof the four samples

paper decoy flowers (see above). The amount of 4-methyl-5-vinylthiazole emitted by each dispenser was calculated between 700–950 μg h^{-1}, similar to the value obtained from a single bagged *Caladium bicolor* inflorescence under the same environmental conditions (Table 1). The traps (91 in total) were installed between 17:30–18:30 h

and retrieved 3–4 h later. Traps with unloaded dispensers were used as paired controls.

Results and Discussion

GC-MS analyses of floral scent samples of the studied species revealed a total of 26 compounds, divided into several chemical classes (see Knudsen et al., (2006) for details): aliphatics (1), benzenoids and phenylpropanoids (9), nitrogen- and/or sulphur-containing compounds (3), terpenoids (10), and miscellaneous cyclic compounds (3) (Table 1). The bouquets of the *Annona* species were each comprised of eight or less compounds, and the bouquet of *Caladium bicolor* of seventeen.

Fragrances of the four species of *Annona* consisted almost entirely of a single compound, 4-methyl-5-vinylthiazole (Fig. 2), which alone accounted for over 97 % of relative flower scent discharge. This sulphur- and nitrogen-containing heterocyclic, reported here for the first time as a floral volatile, also was identified as a major constituent (36.25 %) in samples of the aroid *C. bicolor* (Fig. 3; Table 1).

Derived from the metabolism of amino acids (Schwab et al., 2008), sulphur-containing floral volatiles are found in less than 40 % of the extant angiosperm orders (Knudsen et al., 2006). Among them, thiazoles are largely unknown in flower scents. Recently, two thiazole compounds (2-methylthiazole and 2-methyl-4,5-dihydrothiazole) were reported as minor constituents (≤ 2.5 % relative discharge) in the floral scent of wasp-pollinated *Eucomis* spp. (Hyacinthaceae) (Shuttleworth and Johnson, 2010). Two other compounds, benzothiazole and benzothiazolone, isolated in less than 10 plant species as minor constituents, are actually recognized as ubiquitous environmental contaminants, and their natural occurrence in flowers is subject to controversy (Azizian et al., 2003; Valdes and Zaror, 2006). Unlike sulphides present in the fetid, pungent aroma of bat- or carrion insect-pollinated species (Dobson, 2006), thiazole fragrances commonly emanate notes of tropical fruits, nuts, and green leaves (Maga, 1975; Goeke, 2002). All samples we analyzed emitted a characteristic roasted nut reminiscent aroma, as does pure 4-methyl-5-vinylthiazole.

Most plant volatiles originate from primary metabolism, arising as secondary metabolites of countless feasible alterations of pre-existing biosynthetic pathways (Pichersky et al., 2006; Schwab et al., 2008). We can only speculate about the occurrence of 4-methyl-5-vinylthiazole in flowers. However, while previously unknown as a floral scent volatile, it has been identified as a trace constituent in fruits of *Annona muricata* (Wong and Khoo, 1993; Pino et al., 2001). It also is found in passion fruits (*Passiflora edulis* f. *flavicarpa*, Passifloraceae) (Engel and Tressl, 1991), cupuaçu (*Theobroma grandiflorum*, Malvaceae) (Fischer et al., 1995), and garlic bulbs (*Allium sativum*, Alliaceae) (Yu et al., 1989), a clear indication that several plant lineages bear the necessary biochemical apparatus for its synthesis. Although yet untested, it is plausible that the origin of 4-methyl-5-vinylthiazole in angiosperms is related to the thiazole pathway involved in the multi-enzyme biosynthesis of thiamine (vitamin B1). This compound is an essential co-factor to all living organisms, synthesized *de novo* in all major groups of higher plants (Jurgenson et al., 2009). Through dephosphorylation and dehydration reactions, yet to be elucidated, the precursor of the thiazolic moiety of thiamine, 4-methyl-5-β-hydroxyethylthiazole phosphate, could be converted to 4-methyl-5-vinylthiazole.

The fragrant decoys and traps baited with 4-methyl-5-vinylthiazole installed at both study sites lured exclusively male and female scarab beetles belonging to three species of the genus *Cyclocephala* (Table 2). We did not observe insects approaching unbaited decoy flowers at *Botucatu*, nor

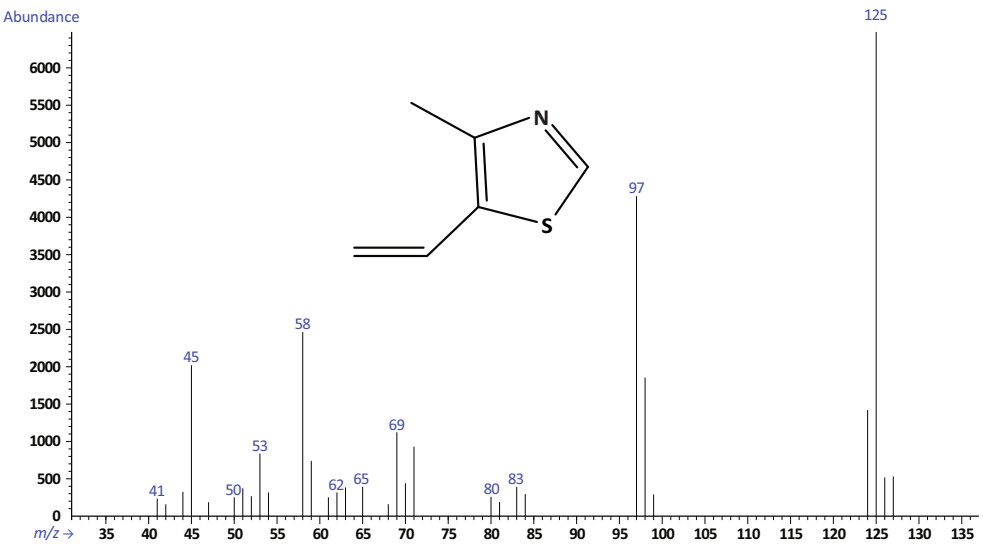

Fig. 2 Quadrupole mass spectra of 4-methyl-5-vinylthiazole (*m/z* 125.0)

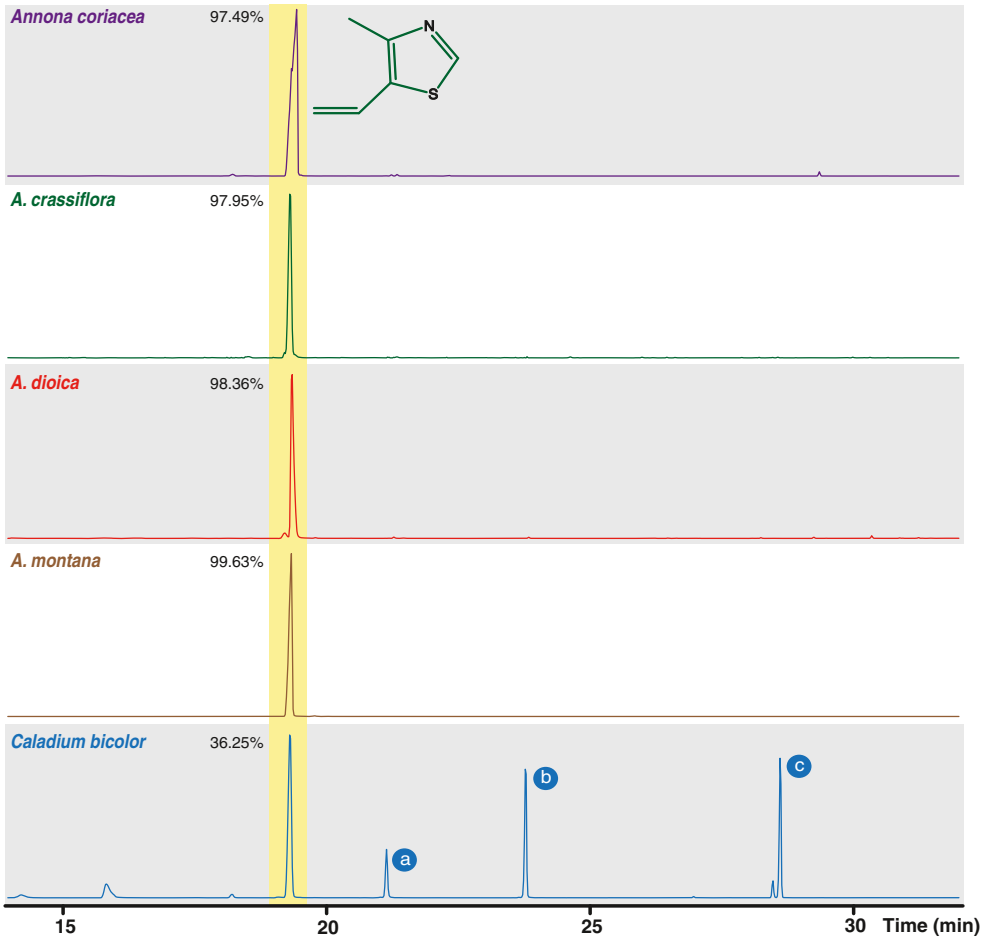

Fig. 3 Chromatograms (total ion counts) of dynamic headspace samples from flowers of four *Annona* species (Annonaceae, magnoliids) and from an inflorescence of *Caladium bicolor* (Araceae, monocots). The peak of 4-methyl-5-vinylthiazole is highlighted and relative percentage of the compound in the overall scent blends is shown for each studied species. Other prominent compounds are: (a) linalool, (b) methyl salicylate, and (c) 1,3,5-trimethoxybenzene

did we recover insects from any of the paired negative control traps at *Igarassu*. Attraction was selective at both sites, as only these three species were recovered out of a wider array of sympatric congeneric species (see Gottsberger (1989) and Maia et al., (2010) for details).

We have gathered evidence of a biochemical convergence between unrelated lineages of angiosperms (Annonaceae and Araceae) towards a specific set of pollinators, either linked to selective expression of a plesiomorphic biosynthetic pathway or to parallel evolution leading to independent floral scent compound production. Convergent evolution in plant metabolism is thoroughly exemplified in plant-pollinator interactions (Pichersky and Lewinsohn, 2011), and sometimes the production of certain floral volatiles can even be assumed as predictors of different pollination syndromes (Dobson, 2006). For example, plants emitting oligosulfides are typically pollinated by carrion/dung flies (Jürgens et al., 2006; Johnson and Jürgens, 2010) or bats (Knudsen and Tollsten, 1995; Von Helversen et al., 2000). Nocturnal plants emitting floral scents rich in lilac aldehydes are associated with noctuid moths (Plepys et al., 2002; Dötterl et al., 2006; Chess et al., 2008).

What is particularly remarkable in our observations is that the attraction of the observed species of *Cyclocephala* towards their flower hosts is highly specific (as aforementioned, other congenerics were not attracted) and linked to the presence of a single compound. This serves as one example of a rarely documented scent-driven 'private communication channel' (Raguso, 2008). A similar scenario was found within reward-based pollination systems involving fragrance seeking male euglossine bees (Apidae, Euglossini) (Whitten et al.,

Table 2 Attraction of pollinator scarab beetles (Scarabaeidae, Dynastinae) of the genus *Cyclocephala* to synthetic 4-methyl-5-vinylthiazole. Field experiments conducted in two localities in Brazil (Botucatu, 22° 52' S, 48°26' W; and Igarassu, 7°49' S, 35°02' W). Installed baited traps were paired to negative controls (unbaited). No insects were recovered from negative controls at either site

	Botucatu	Igarassu
Type of trap	paper flower decoy	'Japanese beetle trap'
# installed traps (baited)	13	91
% traps with captures	46.1	40.7
Total # of captures	26	59
Average captures per trap	2.0	0.65
Species collected (male: female sex ratio)	*C. atricapilla* (1.4: 1)	*C. celata* (1: 1); *C. vestita* (7.2: 1)

1986). The monoterpene *trans*-carvone oxide, common to floral scents of several species of orchids (Kaiser, 1993) and unrelated Euphorbiaceae of the genus *Dalechampia* (Armbruster et al., 1989), *Unonopsis stipitata* (Annonaceae) (Teichert et al., 2009), and *Anthurium* spp. (Araceae) (Schwerdtfeger et al., 2002) induced attractive response of pollinator bees of the genera *Eulaema* and *Euglossa*. The occurrence of *trans*-carvone oxide in angiosperms otherwise is extremely rare (Knudsen et al., 2006), and until now this compound has been isolated solely in plant taxa pollinated by fragrance seeking male euglossine bees. Such a level of chemosensory pollinator selectivity also is exemplified in studies of the diverse, pantropical *Annona*. While 4-methyl-5-vinylthiazole is a dominant element in the scent of the species targeted in our study, it is entirely absent from the bouquet of *Annona glabra*. This species is pollinated instead by small beetles (Chrysomelidae), and its flowers give off a different odor: a sharp, acetone-like fragrance rich in 1,8-cineole, 3-pentanyl acetate and 3-pentanol (Goodrich and Raguso, 2009).

Both *trans*-carvone oxide and 4-methyl-5-vinylthiazole function as main attractive signals for selective subsets of insect visitors, and systems involving these two groups of pollinators show analogous luring strategies (e.g., intense fragrance discharge, high relative amounts in overall blend). Floral scent trails for cyclocephaline scarab beetles, male and female alike, indicate specific micro-niches where they consistently find shelter, reliable food sources, and most importantly, optimal mating opportunities (Gottsberger, 1990). Some authors even speculate that sexual aggregation in anthophilous cyclocephaline scarabs rely on allelochemicals present in floral scents of their preferred host plants (Schatz, 1990; Dieringer et al., 1999; Gibernau et al., 1999). For male euglossine bees, fragrances themselves are coveted rewards used in courtship behavior (Eltz et al., 2005).

It may be that other angiosperms pollinated by cyclocephaline scarabs have similarly adopted 'private communication channel' strategies involving peculiar bioactive compounds, strategically ensuring efficient specific attraction of pollinators. The floral scent of *Cyclanthus bipartitus* (Cyclanthaceae), whose thermogenic inflorescences are visited by two species of *Cyclocephala*, is dominated by an exclusive homoterpene, (*E*)-cyclanthone (> 85 % of the floral blend) (Schultz et al., 1999). Unusual methoxylated esters found in the scent of *Nymphaea lotus* L. (Nymphaeaceae) could well be involved in the attraction of *Rutelorycteres morio*, the effective pollinator of indigenous populations of this water lily in West Africa (Ervik and Knudsen, 2003). The large night-blooming flowers of Central American *Magnolia tamaulipana* and *M. schiedeana*, attractive to *Cyclocephala caelestis* and *C. jalapensis*, respectively (Dieringer and Espinosa, 1994; Dieringer et al., 1999), give off a strong scent dominated by an exclusive methoxylated monoterpene, geranyl methyl ether (Azuma et al., 1999). Methyl 2-methylbutyrate, highly attractive to the pollinator of *Magnolia ovata* (Gottsberger et al., 2012), is more broadly distributed in floral scents, although usually as a minor constituent (Knudsen et al., 2006).

It has been suggested that certain flowering plants exploit pre-existing sensory preferences of specific pollinators (Schiestl, 2010). Floral fragrances might accurately mimic sex pheromones (Schiestl et al., 1999), the scent of preferred oviposition substrates (Stensmyr et al., 2002), and even alarm pheromones of honeybees, an optimal signal for hunting wasps (Brodmann et al., 2009). Both adults and larvae of *Cyclocephala* and other scarab beetles feed on non-floral plant materials (e.g., roots, fruits), to which these insects also might be attracted by olfactory cues (Gottsberger, 1990; Bernhardt, 2000). Attractive compounds present in floral scents of plants pollinated by cyclocephaline scarabs could have been selected through feeding-oriented neuronal preferences of the insects (Schiestl, 2010). Although we do not have evidence that 4-methyl-5-vinylthiazole is part of the innate communication system of *Cyclocephala* beetles, our findings show that it plays a key role in long-range signalling for appropriate feeding and/or mating sites.

Overall, our study led to the identification of 4-methyl-5-vinylthiazole as a floral signal utilized by several *Annona* and an unrelated aroid (*Caladium bicolor*) that convergently attracts their *Cyclocephala* beetle pollinators. Further investigations directed at the biosynthesis of 4-methyl-5-vinylthiazole and its prominence in floral fragrances will show how deep the entanglement of this novel floral scent compound goes into the basis of other lineages of cyclocephaline scarab-pollinated angiosperms.

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Mediation of a Plant-Spider Association by Specific Volatile Compounds

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Abstract *Evarcha culicivora*, an East African jumping spider (Salticidae), is the only spider for which there is evidence of innate olfactory affinity for particular plant species. *Evarcha culicivora* also actively chooses as preferred prey the females of *Anopheles* mosquitoes, and both sexes of *Anopheles* are known to visit plants for nectar meals. Here, we identified compounds present in the headspace of one of these species in Kenya, *Lantana camara*, and then used 11 of these compounds in olfactometer experiments. Our findings show that three terpenes [(*E*)-β-caryophyllene, α-humulene and 1,8 cineole] can be discriminated by, and are salient to, *E. culicivora*. The spiders experienced no prior training with plants or the compounds we used. This is the first experimental demonstration of specific phytochemicals being innately attractive to a spider, a group normally characterized as predators.

Keywords Plant-arthropod interactions · *Evarcha culicivora* · *Lantana camara* · Salticidae · Sensory ecology · Plant volatiles

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Introduction

Many nectar feeding and herbivorous insects associate with particular plant species, and several are known to rely on specific blends of plant-derived volatile compounds for identifying the particular plant species exploited as feeding or oviposition sites (Pichersky and Gershenzon, 2002; Bruce et al., 2005; Waser and Ollerton, 2006; Kessler and Morrell, 2010). That spiders (Araneae) sometimes associate with particular types of plants (e.g., bromeliads: Romero and Vasconcellos-Neto, 2005) might be surprising, as spiders typically are characterized as being predators that feed primarily on insects and other arthropods (Foelix, 2011). Yet numerous examples are now known of spiders also feeding on nectar (Jackson et al., 2001; Taylor and Pfannenstiel, 2008; Meehan et al., 2009), and associating with plants can also reward spiders with opportunities to feed on the insects that visit the plants (Ruhren and Handel, 1999; Whitney, 2004).

Chemoreception is known to have considerable importance for spiders in the context of intraspecific and predator-prey interactions (Pollard et al., 1987; Gaskett, 2007; Nelson and Jackson, 2011). It is customary to distinguish between olfaction and contact chemoreception (Foelix, 1985, 2011), with olfaction depending on the spider detecting specific volatile compounds, or blends of compounds, which are detected by receptors located in the spider's legs and palps (Foelix, 2011). Despite a growing appreciation that spiders may often feed on plant products, little is known about the role of plant-derived volatile compounds in mediating spider-plant relationships. In particular, whether spiders can use volatile cues to identify plants is largely unknown (see Stellwag and Dodson, 2010), although they are able to associate artificial odor with sources of artificial nectar (Patt and Pfannenstiel, 2008).

The East African jumping spider (Salticidae) *Evarcha culicivora* apparently is unique (Jackson and Nelson, 2012) in

being attracted to the odor of cut samples from the plant *Lantana camara* (Verbenaceae; hereafter: *Lantana*) (Cross and Jackson, 2009). Here, our objective was to collect and identify volatile compounds from the headspace of *Lantana* from Kenya and to investigate whether specific environmental chemical cues associated with a plant (*Lantana*) are salient to *E. culicivora*. We provide the first evidence that spiders respond to individual compounds and also discriminate between them. That these compounds are plant-derived is all the more unusual in a group of animals largely known for their predatory behavior.

Methods and Materials

Collection and Identification of Volatile Compounds This work was carried out on the Nairobi campus of the International Centre of Insect Physiology and Ecology (ICIPE). Field portable 65 μm polydimethylsiloxane-divinylbenzene (PDMS/DVB) Merlin Microseal^{TM} 23 gauge needle auto holder blue fibers (Supelco, Bellefonte, PA, USA) were used for all SPME measurements. The fibers were preconditioned at 250 °C for 30 min in a 5890 GC-injection port with the purge valve on, following the manufacturer's instructions. Sampling time was 1 h. After sampling, a SPME fiber was inserted into the injector port of a gas chromatograph fitted with a 4.0 mm gooseneck splitless, taper, liner (Agilent) for a 2 min desorption at 250 °C. After injection, each fiber was cleaned as above, retracted into its holder, and stored for further sampling.

For *in situ* volatile collection, a 10–15 cm long branch of intact *Lantana* was enclosed in a Reynolds® oven bag (Richmond, VA, USA)(482×596 mm) supplied with environmental air (flow rate 260 ml/min) using a portable push-pull field pump (USDA/ARS-CMAVE, Gainesville, FL, USA). The strapped end contained the supply line, vacuum line, and the conditioned SPME fiber. SPME fibers were exposed to the freshly enclosed branch at 0600–0700 h. The oven bags were preconditioned in an oven at 150 °C for 10 min. Methods were the same for *ex situ* volatile collection except that, instead of a living branch, we used a cut branch (length 10–15 cm) from a *Lantana* plant kept damp by cotton wool wrapped with aluminum foil and placed in the glass jar.

SPME fibers were analyzed by GC/MS on a 7890A gas chromatograph (Agilent Technologies, Inc., Beijing, China) coupled to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following conditions: inlet temp 270 °C, transfer line temp of 280 °C, and column oven temperature programmed from 35 to 280 °C with the initial temperature maintained for 5 min, then increased by 10 °C/min to 280 °C, and held at this temperature for 10 min. The GC was fitted with an HP-5 MS low bleed capillary column (30 m×0.25 mm i.d., 0.25 μm) (J&W, Folsom, CA, USA). Helium, at a flow rate of 1.25 ml/min, served as carrier gas. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode. Fragment ions were analyzed over 40–550 m/z mass range in the full scan. The filament delay time was set at zero min.

Compounds were identified by comparing mass spectra and retention index values of our samples to those of authentic compounds. When there was a lack of corresponding reference compounds, structures were proposed on the basis of general ms fragmentation combined with using reference spectra published by library-MS databases (NIST 05, NIST 08). Compositional data were obtained from triplicate measurements of the areas of GC traces due to individual components. We chose 11 of the identified volatiles (Fig. 1) for olfactometer testing with spiders.

General Spider Testing Methods This work was carried out on ICIPE's Thomas Odhiambo Campus in Western Kenya (Mbita Point) using salticids from laboratory cultures (F2 and F3 generation; no prior experience with plants or the compounds used in experiments). No spider individual was tested more than once, and hunger level was standardized by subjecting tests spiders to a 7-d-pre-trial-fast. Rearing methods, as well as the basic procedures used in olfactometer experiments, were as in earlier studies (see: Jackson et al., 2005; Jackson and Nelson, 2012), and only essential details are provided here. Testing was carried out between 0800 h and 1400 h (laboratory photoperiod 12L-12D, lights on at 0700 h) using unmated adult male and female spiders that had matured 2–3 wk beforehand.

Two olfactometer designs were used (retention testing and choice testing). Retention-test olfactometers (Fig. 2) were used to determine how long a test spider would remain in a small holding chamber when exposed to specific odors. Retention tests followed a paired design: each test spider was tested twice over successive days (7 and 8 d of pre-trial fasting), 1 day with an odor source and the other day with a no-odor control (sequence random). In choice tests, each test spider was tested once with two odor sources present at the same time using traditional Y-shaped olfactometers (Fig. 2), with the odor source for each end of the Y being randomized. Airflow in olfactometers was 1500 ml/min (Matheson FM-1000 airflow regulator), and there was no evidence that this airflow setting impaired locomotion or had any adverse effects on *E. culicivora*'s behavior. Between trials, olfactometers were dismantled and cleaned with 80 % ethanol followed by distilled water and were then oven-dried.

Retention Tests During retention tests, air was pushed successively through an odor chamber, a holding chamber, and an exit chamber (see Fig. 2 for dimensions). The test spider was first kept in the glass holding chamber for at least 2 min, with the holding chamber not yet connected to the glass odor and exit chambers. The two ends of the holding chamber were plugged with rubber stoppers. If the test spider was in the half

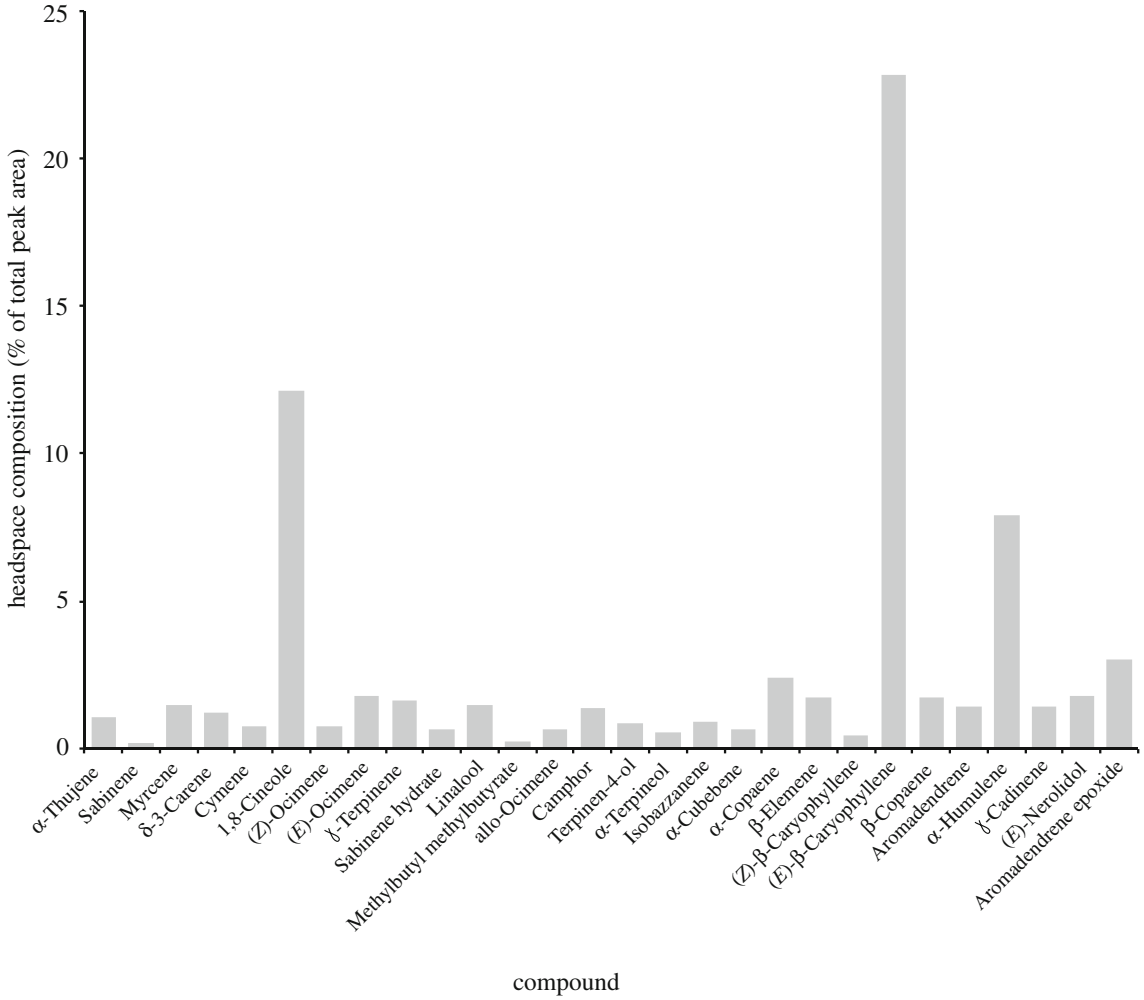


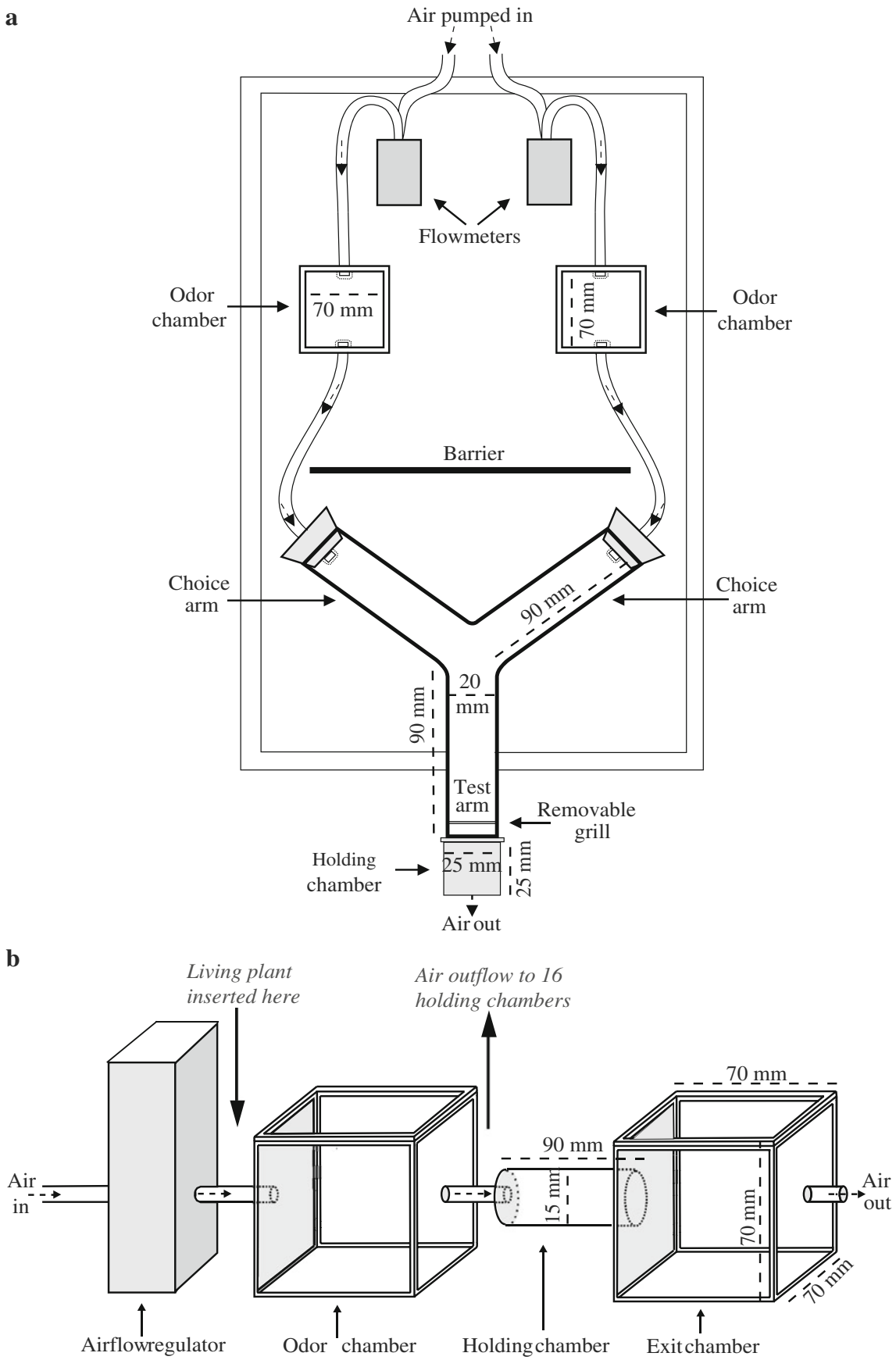
Fig. 1 Percentage of the peak area of compounds found in the headspace of *Lantana camara* from Kenya as determined by GC/MS

of the holding chamber distal to the exit chamber after 2 min, the stoppers were removed, and the holding chamber was placed between the odor and exit chamber. If test spiders were not in the distal portion of the holding chamber at 2 min, we waited until they were in position before connecting the holding chamber to the apparatus and beginning the test. Nylon netting (new netting for each test) prevented spiders from entering the odor chamber, so the only way out of the holding chamber was via the opening into the exit chamber.

Retention tests lasted for a maximum of 60 min. We recorded the test spider's latency to leave the holding chamber (i.e., time elapsing between test beginning and spider entering exit chamber). The spider's latency to leave was recorded as 60 min whenever the test period ended with the spider still in the holding chamber.

The odor sources used during retention testing were either living *Lantana* or preparations of 11 compounds from the headspace of *Lantana* within an inert gelatinous carrier (hereafter referred to simply as 'preparations'). (*E*)-β-Caryophyllene, isolated from natural sources, was purified by flash column chromatography and characterized by ^{1}H NMR and mass spectrometry (Hübner et al., 1997); other volatiles tested were commercial samples. Preparations used as odor sources were placed within the odor chamber. During tests, each odor chamber was supplied with air via its own pump, and 16 of these could be used simultaneously.

Living plants used as odor sources grew in a pot that had been maintained in the field for the previous 2 mo. During testing, the plant was brought into the laboratory and after use returned to the field for 5 d before being re-used. When the odor source was a live plant, the stems, leaves and flowers were enclosed inside a plastic baking bag containing two holes, each fitted with a metal gasket. The air pump was connected with silicone tubing to the bag's inflow hole and the outflow hole connected to an aluminum odor chamber (150×130×130 mm). This chamber had 16 outflow holes, each connected to a flowmeter at the base of 16 separate holding chambers for parallel testing. Controls for live plant tests were identical except that there was no plant between the pump and the aluminum chamber.


a
Air pumped in
Flowmeters
Odor chamber
70 mm
70 mm
Odor chamber
Barrier
Choice arm
90 mm
Choice arm
20 mm
90 mm
Test arm
Removable grill
Holding chamber
25 mm
25 mm
Air out
b
Living plant inserted here
Air outflow to 16 holding chambers
70 mm
90 mm
Air in
15 mm
70 mm
Air out
70 mm
Airflow regulator
Odor chamber
Holding chamber
Exit chamber

◀ **Fig. 2** Glass olfactometers used for: **a** choice testing (view of odor source obstructed by opaque barrier) and **b** retention testing (view of odour source obstructed by black paper taped to outside of odour chamber wall that faced holding chamber). *Dashed arrows* indicate direction of airflow. Not drawn to scale. *Grey italics* indicate modifications made for live plant testing

Preparations were made by adding a prescribed volume of the compound or a blend of compounds to 1.0 g of petroleum jelly (Vaseline^{TM}) in the center of an open glass Petri dish (diam 30 mm). The blends of compounds used in testing were mixtures of (*E*)-β-caryophyllene, 1,8-cineole, and α-humulene in a 2:2:1 ratio by volume (e.g. a 5 μl sample consisted of 2 μl (*E*)-β-caryophyllene, 2 μl 1,8-cineole, and 1 μl α-humulene). These preparations were used for testing 2–7 d after being prepared. When not in use, preparations were wrapped in aluminum foil and refrigerated.

To determine dose-response characteristics we used different amounts of the compounds in the preparations, varying between 4.0 and 0.1 μl. For small volumes of compounds (<1 μl), accurate aliquots were made by first dissolving the compound(s) in paraffin oil and then pipetting 1 μl of the resulting solution into the petroleum jelly (e.g., a 0.5 μl sample of (*E*)-β-caryophyllene was prepared by putting 10 μl of (*E*)-β-caryophyllene in 10 μl of paraffin oil and then blending 1 μl of this solution with 1 g of petroleum jelly). The no-odor control was 1.0 g of petroleum jelly or the 1.0 g of petroleum jelly plus 1 μl of paraffin oil (hereafter, the specified amount of petroleum jelly or petroleum jelly plus paraffin oil will be referred to as the 'carrier'). No preparation of control carrier was used more than once, and the carrier used in the control tests was from the same batch as that used in the paired experimental tests.

The olfactometer was set up 30 min before testing began: all parts of the apparatus were connected; the pump was turned on; the odor source or control carrier was placed within the stimulus chamber, or the potted *Lantana* was covered with the baking bag.

Choice Tests The stem of the Y of choice test olfactometers (Fig. 2) was the 'test arm', and two ends of the Y were the 'choice arms'. Connected to each choice arm, there was a separate glass odor chamber, with one odor source in one chamber and a different odor source in the other. Before testing began, the test spider (N=20 or 25, see Table 1) was confined for 2 min in a holding chamber at the far end of the test arm. While in the holding chamber, the test spider's access to the test arm was blocked by a removable metal 'entrance' grill that fit within a slit in the chamber roof (another grill at the distal end of the holding chamber allowed air to leave). Testing began by lifting the entrance grill. When the spider moved through the test arm, entered a choice arm, and remained there for 30 sec, we recorded the arm entered as the test spider's choice. The spider was allowed 30 min within which to make a choice.

The odor preparations used, and their maintenance during choice testing, were as described for retention tests, but here the carrier on the two sides of the Y-shaped olfactometer was always from the same batch (in retention tests carrier for each pair over successive days was from the same batch).

In addition to the preparations described above, we also tested fresh (used for testing 2–7 d after being made) and old (the same preparation used 25–30 d after being made) preparations to determine whether oxidation or volatilization of odorants affected spider response. Preparations were always fresh unless stated otherwise (Table 1).

We also used living plants (as described for retention testing, except that the outflow holes in the aluminum chamber were connected to the test arms rather than to holding chambers) and plant cuttings as odor sources. The plant cutting for any given odor chamber was two umbels (median weight/umbel (1st and 3rd quartiles), 364 (329 and 384) mg, N=10) taken from *Lantana* in the field at 0630 h, used repeatedly for a single day and then discarded.

Data Analysis In all retention tests, we used both adult males and females (N=25 for each sex). A difference score was calculated for each test spider by subtracting the latency to leave the holding chamber when tested with odor from its latency to leave the holding chamber when tested with control. The resulting scores were positive when the spider spent more time in the holding chamber when tested with odor, and negative when the spider spent more time in the holding chamber when tested with the control.

We tested for sex differences for each preparation and amount of odorant using Mann-Whitney tests, as these data often failed to meet the assumptions required for parametric analyses. In all cases, we found no sex-based differences on the difference scores (Table 2) and pooled the original data for males and for females for subsequent testing. There was also no difference between males and females (U=312.5, P=0.992) when tested with the odors from living *Lantana,* and data for both sexes were pooled. The median score (1st and 3rd quartiles) for both females and males was 28.0 min (5.0 and 43.0).

For each amount of each preparation, we then ran Wilcoxon matched-pairs signed rank tests on latency to exit the holding chamber during control and experimental trials (null hypothesis: latency to leave the holding chamber when tested with odor source matched that in control trials). To test for overall differences in response to preparation amounts we ran Kruskal-Wallis tests on the difference scores from pooled data from males and females, and compared between amounts using Dunn's multiple comparison tests. Choice-test data from individual treatments in the Y-shaped olfactometer were analyzed using binomial tests (H_o=50:50).

Table 1 Choice testing of *Evarcha culicivora* in Y-shaped olfactometer. Binomial tests comparing the number of spiders that chose odor 1 compared with odor 2

Odor 1	Odor 2	Chose 1	Chose 2	Chose 1 (%)	P	Preference
(*E*)-β-Caryophyllene 1.0	(*E*)-β-Caryophyllene 4.0	12	13	48	1.000	NS
(*E*)-β-Caryophyllene 1.0	(*E*)-β-Caryophyllene 0.5	18	7	72	0.043	(*E*)-β-Caryophyllene 1.0
(*E*)-β-Caryophyllene fresh	(*E*)-β-Caryophyllene old	11	14	44	0.690	NS
(*E*)-β-Caryophyllene 4.0	α-Humulene 4.0	13	12	52	1.000	NS
(*E*)-β-Caryophyllene 4.0*	α-Humulene 4.0	11	14	44	0.690	NS
(*E*)-β-Caryophyllene 1.0	α-Humulene 1.0	14	11	56	0.690	NS
(*E*)-β-Caryophyllene 1.0*	α-Humulene 1.0	10	15	40	0.424	NS
(*E*)-β-Caryophyllene 0.5	α-Humulene 0.5	19	6	76	0.015	(*E*)-β-Caryophyllene 0.5
(*E*)-β-Caryophyllene 0.5*	α-Humulene 0.5	14	11	44	0.690	NS
(*E*)-β-Caryophyllene 4.0	1,8-Cineole 4.0	16	4	80	0.012	(*E*)-β-Caryophyllene 4.0
(*E*)-β-Caryophyllene 1.0	1,8-Cineole 1.0	15	5	75	0.042	(*E*)-β-Caryophyllene 1.0
(*E*)-β-Caryophyllene 1.0*	1,8-Cineole 1.0	17	3	85	0.003	(*E*)-β-Caryophyllene 1.0
(*E*)-β-Caryophyllene 0.5	1,8-Cineole 0.5	17	3	85	0.003	(*E*)-β-Caryophyllene 0.5
(*E*)-β-Caryophyllene 0.5*	1,8-Cineole 0.5	15	5	75	0.042	(*E*)-β-Caryophyllene 0.5
(*E*)-β-Caryophyllene 1.0	Blend 1.0	18	7	72	0.043	(*E*)-β-Caryophyllene 1.0
(*E*)-β-Caryophyllene 1.0	Cut *Lantana* sample	13	12	52	0.841	NS
(*E*)-β-Caryophyllene 1.0*	Cut *Lantana* sample	16	9	64	0.161	NS
α-Humulene 1.0	α-Humulene 0.5	16	4	80	0.012	α-Humulene 1.0
α-Humulene 1.0	α-Humulene 4.0	12	13	48	1.000	NS
α-Humulene fresh 1.0	α-Humulene old 1.0	8	12	40	0.371	NS
α-Humulene 1.0	Blend 1.0	18	7	72	0.043	α-Humulene 1.0
α-Humulene 1.0	Cut *Lantana* sample	9	11	45	0.824	NS
α-Humulene 1.0*	Cut *Lantana* sample	11	9	55	0.824	NS
α-Humulene 1.0	1,8-Cineole 1.0	17	3	85	0.003	α-Humulene 1.0
α-Humulene 1.0*	1,8-Cineole 1.0	20	0	100	< 0.001	α-Humulene 1.0
1,8-Cineole 1.0	1,8-Cineole 4.0	14	11	56	0.690	NS
1,8-Cineole 1.0	1,8-Cineole 0.5	18	7	72	0.043	1,8-Cineole 1.0
1,8-Cineole 1.0	Blend 1.0	22	3	88	<0.001	1,8-Cineole 1.0
1,8-Cineole fresh 1.0	1,8-Cineole old 1.0	12	13	48	1.000	NS
Cut *Lantana* sample	1,8-Cineole 1.0	20	5	80	0.004	Cut *Lantana* sample
Cut *Lantana* sample	Blend 1.0	21	4	84	<0.001	Cut *Lantana* sample
Blend 5.0	(*E*)-β-Caryophyllene 1.0	15	10	60	0.424	NS
Blend 5.0	α-Humulene 1.0	14	11	56	0.690	NS
Blend 5.0	1,8-Cineole 1.0	19	6	76	0.015	Blend 5.0
Blend 5.0	Blend 1.0	20	5	80	0.004	Blend 5.0
Blend 5.0	Cut *Lantana* sample	13	12	52	1.000	NS
Living *Lantana*	Cut *Lantana* sample	13	12	52	1.000	NS
Living *Lantana**	Cut *Lantana* sample	10	15	40	0.424	NS

Numbers beside each odor denote the amount used (μl)

Males only tested unless marked with * (female trials)

Blend: blend of (*E*)-β-caryophyllene, 1,8-cineole and α-humulene

NS not significant

Results

Identification of Volatile Compounds Regardless of whether sampling was from a living plant or a cut sample from a plant, our analyses revealed that the same three volatile compounds showed the largest GC trace peak areas (Fig. 1). These were two sesquiterpenes, (*E*)-β-caryophyllene (intact 22.8%of total peak area; cut 37.9 % of total peak

Table 2 Descriptive statistics and Mann-Whitney tests (*df*=48 for all) on the difference scores (see text for details) for retention testing of male and female *Evarcha culicivora* with differing amounts of several compounds found in the headspace of *Lantana camara.* Blend: blend of (*E*)-β-caryophyllene, 1,8-cineole, and α-humulene

Compound	Amount (μl)	Median score (1st; 3rd quartile) females	Median score (1st; 3rd quartile) males	U value	P
(*E*)-β-Caryophyllene	4.0	25.0 (7.0; 37.0)	25.0 (4.0; 34.0)	292.5	0.705
	1.0	18.0 (10.0; 36.5)	17.0 (4.5; 30.0)	260.0	0.313
	0.5	14.0 (6.0; 33.0)	13.0 (3.5; 28.0)	271.0	0.426
	0.1	1.00 (−9.0; 8.0)	1.00 (−7.5; 6.0)	297.5	0.778
α-Humulene	4.0	26.0 (5.0; 49.5)	17.0 (0.0; 36.0)	250.0	0.229
	1.0	32.0 (11.5; 51.0)	21.0 (10.5; 35.5)	250.5	0.233
	0.5	10.0 (−0.5; 40.5)	15.0 (1.5; 27.5)	310.5	0.977
	0.1	0.0 (−14.0; 12.0)	1.00 (−9.0; 7.5)	298.0	0.786
1,8-Cineole	4.0	8.0 (1.0; 45.0)	9.0 (−1.0; 30.0)	287.0	0.627
	1.0	14.0 (−6.5; 44.5)	10.0 (1.5; 30.0)	285.0	0.600
	0.5	16.0 (0.5; 28.0)	13.0 (1.5; 23.0)	300.5	0.823
	0.1	0.0 (−6.0; 10.0)	−1.00 (−7.5; 9.5)	306.0	0.907
Blend	1.0	1.0 (−5.5; 12.0)	1.0 (−9.0; 8.5)	312.0	1.000
	5.0	20.0 (7.5; 48.0)	20.0 (8.0; 45.0)	298.0	0.786
α-Terpineol	1.0	3.0 (−8.0; 13.0)	0.0 (−6.0; 7.0)	288.0	0.641
Aromadendrene	1.0	0.0. (−4.5; 10.0)	0.0 (−8.5; 6.5)	282.5	0.567
δ-3-Carene	1.0	5.0 (−0.5; 11.0)	0.0 (−8.0; 7.0)	244.9	0.186
Linalool	1.0	1.0 (−6.5; 4.0)	3.0 (−8.5; 10.0)	247.0	0.206
Myrcene	1.0	−2.0 (−14.0; 7.0)	0.0 (−3.0; 5.0)	275.0	0.472
E-Nerolidol	1.0	0.0 (−7.0; 9.5)	0.0 (−14.0; 4.5)	264.5	0.356
E-Ocimene	1.0	1.0 (−11.0; 9.5)	1.0 (−8.0; 7.0)	302.0	0.846
α-Thujene	1.0	1.0 (−10.5; 6.0)	1.0 (−6.0; 5.0)	308.0	0.938

area) and α-humulene (intact 7.9 % of total peak area; cut 11.3 % of total peak area), and a monoterpene, 1,8-cineole (intact 12.1 % of total peak area; cut 15.3 % of total peak area).

Retention Tests As all compounds and blends were tested at a volume of 1.0 μl, these were the data on which we ran a Kruskal-Wallis test, within which we also used the data for living *Lantana*. There was a significant overall effect of treatment (H_{12}=127.1, P<0.001), with (*E*)-β-caryophyllene, α-humulene, and 1,8-cineole and living *Lantana* not significantly differing from each other and all eliciting significantly more positive scores than the rest of the compounds tested (Fig. 3). There was no difference between the latency to leave the holding chamber in control and experimental tests when tested with samples of α-terpineol (W=120.0, P=0.541), aromadendrene (W=81.0, P=0.672), δ-3-carene (W=239.0, P=0.193), linalool (W=94.0, P=0.611), myrcene (W=−59.0, P=0.726), *E*-nerolidol (W=−61.0, P=0.726), *E*-ocimene (W=−6.0, P=0.981), or α-thujene (W=42.0, P=0.833).

When tested with the odor from living *Lantana*, spiders spent significantly more time in the holding chamber with the odor of *Lantana* (W=1054, P<0.001) than they did with the no-odor control. With the three principal compounds shown to be salient to the spider, we carried out retention tests using not only a volume of 1.0 μl but also higher and lower volumes. A threshold response to the salient compounds was observed. The latency to leave the holding chamber with odor of (*E*)-β-caryophyllene (Fig. 4a) was significantly longer than the control at amounts ≥0.5 μl (4.0 μl, W=1057, P<0.001; 1.0 μl, W=1105, P<0.001; 0.5 μl, W=990.0, P<0.001; 0.1 μl, W=46, P=0.810). The same effect was seen with α-humulene (4.0 μl, W=1075.0, P<0.001; 1.0 μl, W=1080.0, P<0.001; 0.5 μl, W=939.0, P<0.001; 0.1 μl, W=39, P=0.836; Fig. 4c), and 1,8-cineole (4.0 μl, W=866.0, P<0.001; 1.0 μl, W=783.0, P=0.001; 0.5 μl, W=854.0, P<0.001; 0.1 μl, W=19, P=0.929; Fig. 4e).

The volume of the preparation used had an overall effect on spider response with (*E*)-β-caryophyllene (W_3=38.64, P<0.001), α-humulene (W_3=38.32, P<0.001), and with 1,8-cineole (W_3=19.27, P<0.001). The pattern was similar for each of these compounds; namely, spider response did not differ between volumes of 4.0, 1.0, and 0.5 μl, and that all of these elicited significantly higher retention latencies (in all cases P<0.01) than preparations at an amount of 0.1 μl (Fig. 4b, d, f).

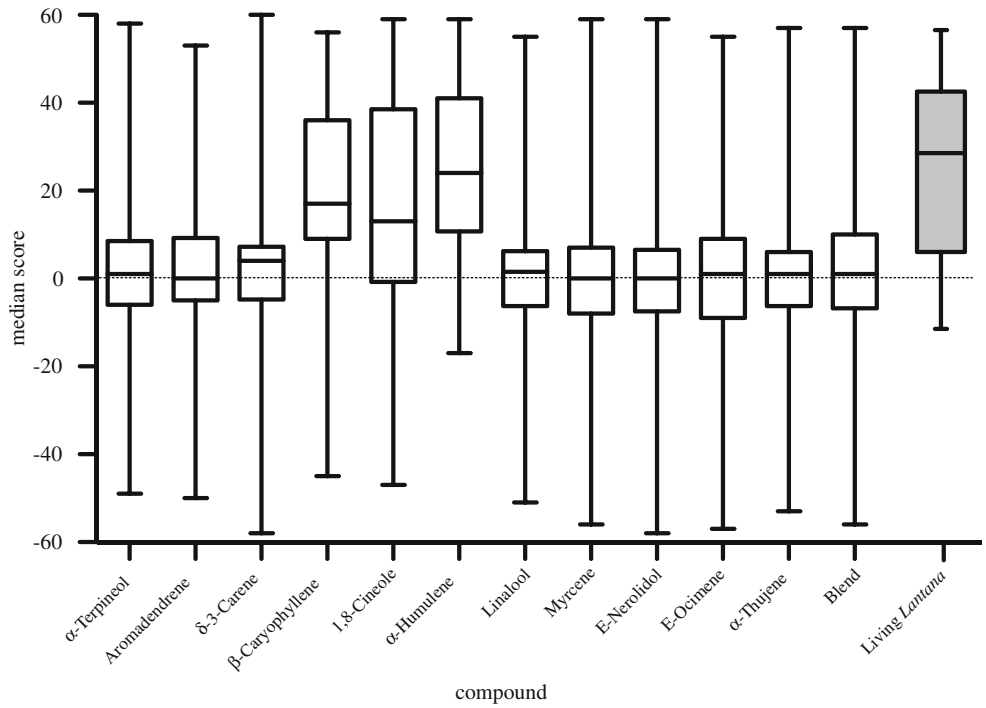


Fig. 3 Boxplots (median and quartiles) with whiskers (min and max) of difference score of retention time with living *Lantana camara* (shaded) and with different compounds found in the headspace of *L. camara*. Scores calculated by subtracting latency to leave holding chamber when tested with control from latency to leave holding chamber when tested with 1.0 μl of odor or with a live plant (positive: spider spent longer within holding chamber with odor; negative: spider spent longer within holding chamber with control). Dunn's pairwise comparisons showed that only cineole, (*E*)-β-caryophyllene, α-humulene and *L. camara* had any significant differences to the rest of the compounds. Other than *L. camara,* (*E*)-β-caryophyllene and 1,8-cineole all compounds were significantly different from α-humulene ($P<0.001$). Other than *L. camara* and 1,8-cineole, all other compounds were significantly different ($P<0.001$) from (*E*)-β-caryophyllene, except δ-3-carene ($P<0.01$). Other than *L. camara,* (*E*)-β-caryophyllene, α-humulene, aromadendrene, δ-3-carene and the blend all compounds except were significantly different ($P<0.05$) from 1,8-cineole, except myrcene ($P<0.01$)

The latency to leave the holding chamber with the blended preparation was significantly longer than the control at 5.0 μl ($W=1152$, $P<0.001$), but not at 1.0 μl ($W=112.0$, $P=0.569$) (Fig. 5a). Using Mann-Whitney tests, the combined male and female difference scores were then compared between volumes to see if volume had an effect on behavior. Spiders spent significantly longer in the retention chamber at an amount of 5.0 μl than at 1.0 μl ($U=458.0$, $P<0.001$; Fig. 5b).

Choice Tests Unlike in retention tests where each spider was tested with only one odor source, spiders were exposed to two odor sources simultaneously in choice tests, the rationale of this testing design being to ascertain the spider's ability to discriminate between compounds and its preferences when given a choice between them. The results of the choice tests were consistent with the pattern established with the retention tests and provided further details about some of the responses. In choice tests, *E. culicivora* chose the odor of (*E*)-β-caryophyllene over each of the individual volatiles that retention testing established were not salient to *E. culicivora* (Table 3). Choice tests were used to determine dose-response effects using the three salient compounds. For each of these compounds, there was no significant difference in volume of compound chosen when the volume of each compound was ≥1 μl, but spiders chose these higher volumes significantly more often when the alternative was a compound at a volume of 0.5 μl.

To ascertain whether aging preparations diminished their effectiveness at attracting *E. culicivora*, we carried out choice tests with the three compounds shown to be salient in retention testing, with the spider's choice being between fresh and old preparations. We found no significant difference between how many spiders chose one instead of the other. When choice tests were carried out between living plants and cut samples of *Lantana,* no difference in the number of spiders choosing one or the other was found.

When cut samples of *Lantana* were paired with either (*E*)-β-caryophyllene or α-humulene in a volume more than 0.5 μl, the number of test spiders that chose *Lantana* was not significantly different from the number of test spiders that chose the synthetic preparation. However, significantly more test spiders chose *Lantana* than chose 1,8-cineole at a volume of 1 μl preparation. They also chose both (*E*)-β-

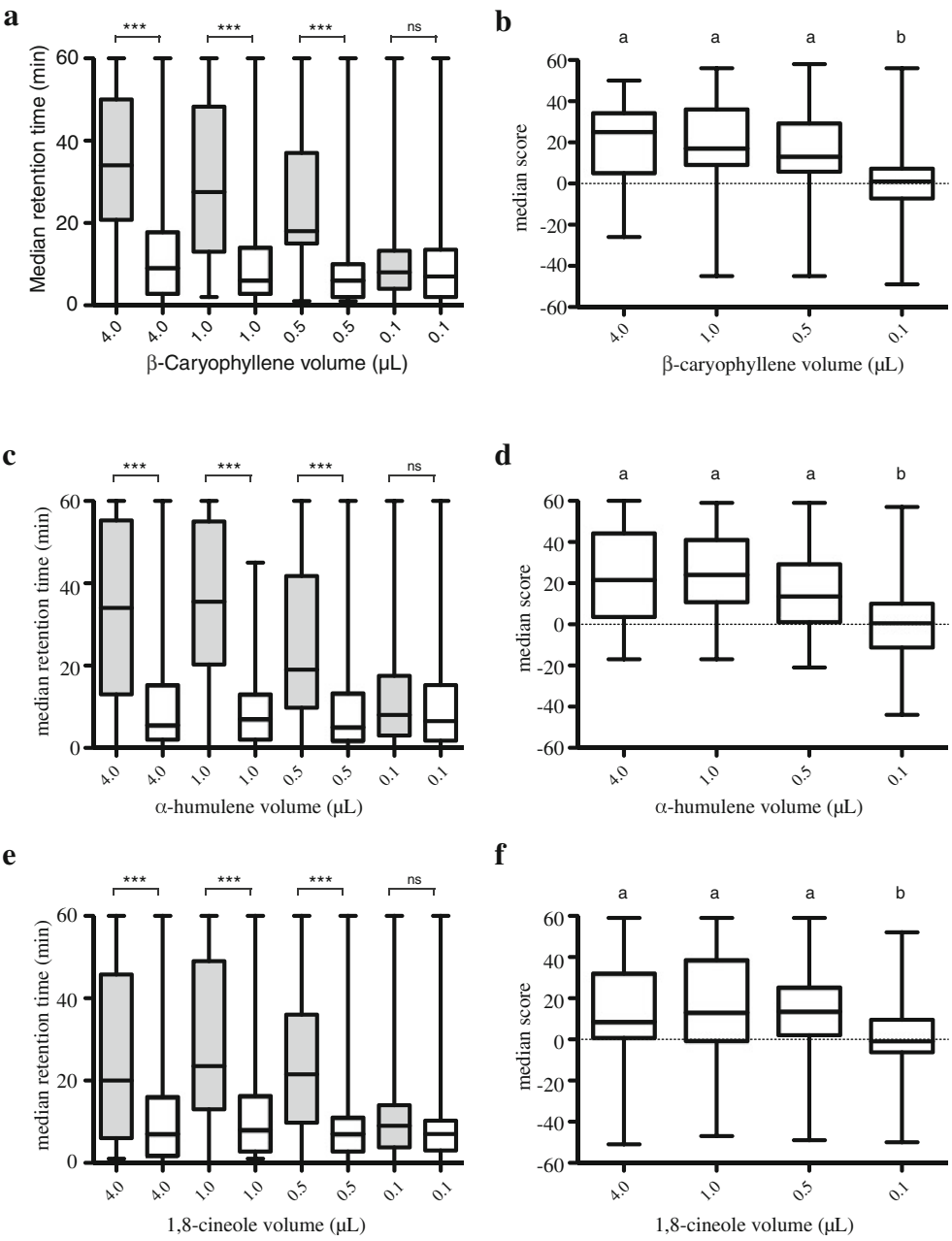


Fig. 4 Boxplots (median and quartiles) with whiskers (min and max) for retention testing with three salient compounds found in the headspace of *Lantana camara*. **a** Time *Evarcha culicivora* spent in holding chamber in the odor (experimental) and paired control treatments with different amounts of (*E*)-β-caryophyllene. **b** Difference score of retention time with different amounts of (*E*)-β-caryophyllene. Scores calculated by subtracting latency to leave holding chamber when tested with control from latency to leave holding chamber when tested with odor of (*E*)-β-caryophyllene (see Fig. 1 for details). **c** Time spent in holding chamber with different amounts of α-humulene and matched controls. **d** Difference score of retention time with different amounts of α-humulene. **e** Time spent in holding chamber with different amounts of 1,8-cineole and matched controls. **f** Difference score of retention time with different amounts of cineole. $N=50$. ***$P<0.001$. *Letters* denote significant differences (all $P<0.01$). In (**a**) (**c**) and (**e**) *shaded boxes* denote experimental treatments and *unshaded* denote controls

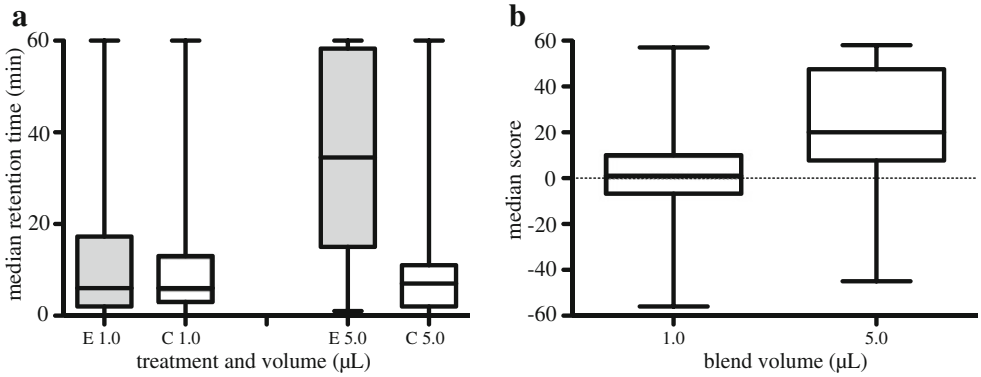


Fig. 5 Boxplots (median and quartiles) with whiskers (min and max) for retention testing with blend preparations. **a** Time *Evarcha culicivora* spent in holding chamber with different amounts of blend preparations and matched controls. **b** Difference score (see Fig. 1 for details) of retention time with different amounts of blend preparations. $N=50$

Table 3 Choice testing of *Evarcha culicivora* in Y-shaped olfactometer using 1 μl of (*E*)-β-caryophyllene (odor 1) as a representative salient odor compared with compounds that retention testing suggested were not salient to spiders (odor 2). N=25 for each sex, no differences between sexes (χ^2 test of independence) so sexes pooled. In all cases spiders chose (*E*)-β-caryophyllene significantly more often (P<0.001) than the alternative (binomial tests)

Odor 2	Chose 1	Chose 2	Chose 1 (%)
α-Terpineol	39	11	78
Aromadendrene	38	12	76
δ-3-Carene	39	11	78
Linalool	41	9	82
Myrcene	39	11	78
E-Nerolidol	40	10	80
E-Ocimene	43	7	86
α-Thujene	45	5	90

caryophyllene and α-humulene more often than equal volumes of cineole at the concentrations tested. At higher volumes, there was no significant tendency to choose when the compound was either (*E*)-β-caryophyllene or α-humulene. However, when the volume was 0.5 μl, the number of spiders that chose (*E*)-β-caryophyllene was significantly more than the number that chose α-humulene.

Significantly more test spiders chose preparations of (*E*)-β-caryophyllene, α-humulene, or 1,8-cineole alone than chose the blend at a volume of 1.0 μl, but there was no significant difference in how many test spiders chose the blend at a volume of 5.0 μl than chose (*E*)-β-caryophyllene or α-humulene. However, more spiders chose the 5.0 μl blend than chose 1,8-cineole.

Discussion

Evarcha culicivora is the only predator known to feed indirectly on vertebrate blood by actively choosing blood-carrying mosquitoes as prey (Jackson et al., 2005). *Evarcha culicivora* is also of unusual interest in the context of malaria because it targets *Anopheles* as preferred mosquitoes, this being the genus to which all vectors of human malaria belong (Jackson and Nelson, 2012). However, there is another context in which this East African predator is unusual. A previous study (Cross and Jackson, 2009) showed that the odors of *Lantana camara* and *Ricinus communis* are salient to *E. culicivora*. This is the only documented example of a spider having an olfactory affinity for particular plant species.

Here, we extend this observation by identifying specific chemicals present in the head space of *L. camara* in Kenya and elucidating the response of *E. culicivora* to 11 of these volatiles. From olfactometer experiments, we now have evidence that, in contrast to the other chemicals tested, the three principal volatile components are salient to *E. culicivora*. Our findings further show that *E. culicivora*'s response to these particular compounds, the sesquiterpenes (*E*)-β-caryophyllene and α-humulene, and a monoterpene 1,8-cineole, is comparable to their response to the more complex odor blend from *Lantana,* and is not sex-specific. As second-generation laboratory-reared spiders that had not been exposed to plants or to the compounds we used, we characterize *E. culicivora*'s responses as innate. This is the first experimental evidence of any spider species expressing an innate affinity for specific plant-derived volatile compounds.

The attractant (*E*)-β-caryophyllene is a natural product found in a wide range of plant species (Knudsen et al., 2006). (*E*)-β-Caryophyllene is the most abundant volatile in essential oils from *L. camara* grown in Cameroon and Madagascar (Ngassoum et al., 1999), and plentiful in plants grown in Brazil and India (da Silva et al., 1999; Khan et al., 2002). α-Humulene is commonly found alongside (*E*)-β-caryophyllene (van der Hoeven et al., 2000; Abel et al., 2009). The monoterpene 1,8-cineole was detected in the headspace of all our plant samples. Of these three compounds, responses of *E. culicivora* were stronger to the sesquiterpenes, α-humulene and especially (*E*)-β-caryophyllene.

With some plants, the release of (*E*)-β-caryophyllene is associated with damage by herbivores (e.g., Turlings et al., 1998; Abel et al., 2009) and appears to play a role in plant defense against herbivore attack. This can occur through the deterrence of arthropods (including possibly one spider, Junker et al., 2011), or through its role in tri-trophic signaling, in which herbivore-induced plant volatiles attract natural enemies of the herbivores causing damage to the signaling plant (e.g., Rasmann et al., 2005; Kessler and Morrell, 2010). Since there was no significant difference in the response of *E. culicivora* to volatiles released by intact plants and cut samples, plant damage does not appear to be a pertinent factor in this case.

For a variety of parasitic insects (e.g., Weissbecker et al., 1999; Park et al., 2001; Bichăo et al., 2003; Schmidt-Büsser et al., 2011), at least a weak electroantennogram response can be elicited by exposure to (*E*)-β-caryophyllene, but the strongest behavioral responses tend to occur when the insect is exposed to a mixture of terpenes instead of individual terpenes by themselves (e.g., Jallow et al., 1999; Tasin et al., 2006). Our findings for *E. culicivora* are unusual because there is no evidence of response to mixtures differing from response to the individual component compounds.

It is likely that the function of *E. culicivora*'s affinity for specific plant-derived compounds is multifaceted. Finding the source of these plant volatiles might, for example, assist in the locating of food (either plant products or mosquitoes). Although spiders are often characterized as being

exclusively predatory, many species are better described as omnivorous predators that supplement their diet with nectar (Pollard et al. 1995; Taylor and Pfannenstiel, 2009). In particular, many salticids (Jackson et al., 2001; Meehan et al., 2009), including *E. culicivora* (unpubl.), feed on nectar.

It is well established that both male (Clements, 1999) and female mosquitoes, including *Anopheles*, frequently visit plants for nectar meals (Yuval, 1992; Foster, 1995); the plants visited include *L. camara* (Impoinvil et al., 2004; Manda et al., 2007). The odorants that attract mosquitoes to particular plants are poorly understood, but it has been shown that tsetse flies are attracted to the odor of *Lantana*. As both sexes of tsetse flies feed solely on blood, these insects probably visit to plants primarily for shelter. However, it is of interest that tsetse flies have receptors on their antennae that are especially responsive to (*E*)-β-caryophyllene (Syed and Guerin, 2004).

It is tempting to propose that finding the source of specific plant compounds facilitates *E. culicivora*'s encounters with preferred prey. However, the complexity of this system makes it likely that such a link would be indirect, rather than direct in nature. For example, as diurnal hunters they may seldom encounter *Anopheles* on plants, as they tend to be nocturnal. If attraction by plant compounds functions in locating mosquitoes as prey, it might be as a means by which the predator arrives in the general vicinity of habitats where the predator might find the resting mosquito on other substrates, such as the walls of houses. The role of *Lantana* in the biology of *E. culicivora* is currently poorly understood, but the identification of specific compounds, notably (*E*)-β-caryophyllene, that influence this spider's behavior promises to be an important step in ongoing research.

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Diaspore Trait Preferences of Dispersing Ants

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Abstract Elaiosomes of myrmecochorous plant seeds are known to enhance the attraction of diaspore-dispersing ants by serving as a nutritional reward. However, it remained unclear which (nutritional) compounds affect diaspore preferences of ants. We hypothesized that apart from elaiosome/seed-size ratio, volume, and physical surface of diaspores, the quantity and the composition of fatty acids, amino acids, and sugars strongly influence the diaspore preferences of different species. Chemical (nutritional) profiles as well as structural properties of seeds with and without elaiosomes were analyzed and correlated with observed seed choice behavior of ants. Cafeteria experiments in the field confirmed the enhanced attractiveness of elaiosome-bearing seeds for all three ant species tested (*Lasius fuliginosus*, *Myrmica ruginodis*, and *Temnothorax nylanderi*), although seeds lacking elaiosomes also were transported. In multiple-choice cafeteria experiments with simultaneously offered diaspores of 16 plant species with and without elaiosome and with highly varying structural and chemical properties, all three ant species showed distinct preferences for certain diaspore species. Correlation analyses confirmed that the presence of an elaiosome represents the crucial factor that favors ant diaspore dispersal. In addition, the composition and the content of free amino acids, and to varying degrees fatty acids, were found to significantly affect preferences of each ant species, whereas the effect of single fatty acids acting as chemical triggers for diaspore transport by ants, as supposed by several studies, was not confirmed. In conclusion, although at least some diaspore species lacking elaiosomes attract ants for diaspore removal services by presenting nutritional seed coats, the production of elaiosomes seems to provide a worthwhile investment. Elaiosomes ensure rapid diaspore detection and removal due to chemical cue compounds and by offering a highly nutritional food supply, probably fitting the nutritional demands of ants.

Keywords Ants · Elaiosomes · Nutrients · Diaspore dispersal · Diaspore transport

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Introduction

Myrmecochory is a worldwide phenomenon with its maximum in the Mediterranean regions (Beattie, 1985). A recent review stated that myrmecochory is present in at least 4.5 % of all species, in 2.5 % of all genera, and in 17 % of all families of angiosperm plants, with at least 101 independent origins (Lengyel et al., 2010). In Central Europe, seeds with attached food bodies (elaiosomes) are mainly found among herbaceous plants growing in the understorey of deciduous forests, flowering in early spring. Such elaiosomes attract a large number of ant species and serve as rewards for dispersal services (Beattie, 1985), probably due to their rich content in essential and easily accessible nutrients (Bresinsky, 1963; Gammans et al., 2005).

Since the term 'myrmecochory' was coined (Sernander, 1906), many studies have dealt with the myrmecochorous syndrome, describing several different crucial factors that may determine the efficiency of this mutualism. The size of seeds and elaiosomes has been shown to affect diaspore acceptance of dispersing ants with strong preferences for high elaiosomes/seed-size ratios (Oostermeijer, 1989; Hughes and Westoby, 1992; Mark and Olesen, 1996). Ants carry diaspores to their nest where they feed the elaiosome to their brood (Handel and Beattie, 1990) and discard the remaining intact seed to a refuse pile that is generally regarded to offer optimal growing conditions (Beattie, 1985). Depending on the size of the diaspore and the mandible-span of the ant, elaiosomes may serve primarily as handles that facilitate diaspore-transportation by ants (Gorb and Gorb, 1999a). Elaiosome removal leads to decreased ants' interest towards seeds and higher rejection rates (Servigne and Detrain, 2010), but also to enhanced seedling emergence and reduced long-term seed predation (Garrido et al., 2009). In the ants' nests, it depends on the behavior of each ant species how the respective myrmecochorous diaspore is processed. Competing dynamics of ant- and plant-specific elaiosome detachment and ant-specific seed rejection lead to variable outcomes of dispersed items and nutritional benefit to the ants, with intact elaiosome-attached seeds rejected and detached seeds remaining in the nests (Servigne and Detrain, 2010).

Some authors have speculated about the existence of a chemical signal that acts as a trigger for ant dispersal behavior. They consider oleic acid (e.g., Marshall et al., 1979; Pfeiffer et al., 2010) or its dimer diolein (Skidmore and Heithaus, 1988; Brew et al., 1989) as the crucial component of myrmecochory inducing seed carrying behavior. Both compounds have been assumed to trigger 'corpse carrying behavior' (Wilson et al., 1958; Hughes et al., 1994) leading to the conclusion that elaiosomes are dead insect analogues (Carrol and Janzen, 1973; Hughes et al., 1994).

Each of these studies on the mechanisms of myrmecochory presents a single structural or chemical factor driving the interaction between one ant species and one plant species. However, it may be too one-dimensional to expect a single component to be responsible for such a complex and generalized mutualism (Mayer et al., 2005). More recently, a study on the nutritional profiles of seeds and elaiosomes of several plant species pointed out the key role of the availability of nutrients of elaiosomes for the interaction between myrmecochorous plants and ants (Fischer et al., 2008). They concluded that the chemical composition of diaspores may fit the physiological demands of ant colonies, especially in seasons of reduced food availability, e.g., essential amino acids (Fischer et al., 2008), monosaccharides (Fischer et al., 2008), and poly-unsaturated fatty acids (Gammans et al., 2005; Fischer et al., 2008). So far, complementary experiments that combine comprehensive chemical diaspore analyses with ant bioassays have not been done.

In the present study, we combined chemical analyses of diaspores with ant preference tests. Our aim was to determine factors that regulate the interaction of three different ant species with elaiosome-bearing seeds and those lacking elaiosomes. We hypothesized that the seed choice of each ant species would be affected by various structural and chemical diaspore characteristics. Chemical cue compounds in elaiosomes may trigger diaspore detection, whereas nutritional diaspore profiles may affect the ants' choice for diaspores most suitable fitting their physiological demands.

We addressed the following questions: 1) Are ants selective among a broad range of different diaspores with and without elaiosomes? 2) Do structural and/or chemical parameters of diaspores with and without elaiosomes correlate with preferences of ants? 3) Do nutritional diaspore profiles and/or chemical triggers affect the ants' choice?

Methods and Materials

Ants The studied ants belonged to the species *Lasius (Dendrolasius) fuliginosus* (LATREILLE 1798), *Myrmica ruginodis* (NYLANDER 1846), and *Temnothorax nylanderi* (FÖRSTER 1850). *Lasius fuliginosus* colonies consist of up to 2 million workers. Their cardboard nests are soaked with honeydew and stabilized by fungal hyphae, and they occur in the trunk base of trees, often scattered among several tree individuals. *Lasius fuliginosus* can build long and wide chemically marked foragers trails, they nourish on trophobiosis with aphids and coccids, or their diet is zoophagous or necrophagous (Seifert 2007). *Myrmica ruginodis* also has large colonies with multiple queens and large numbers of workers. Nests are located in the ground, under stones, in dead wood, or cushion plants or mound-shaped. Their diet is zoophagous or trophobiotic; they also feed on fleshy fruits and nectar (Seifert 2007). Comparably small colonies of *T. nylanderi* with up to 90 workers often occur in high densities. Their nests are located in tree trunks, bark, or hollow acorns, nuts, or galls. The diet of *T. nylanderi* is mainly zoophagous, never trophobiotic, but they also feed on honey dew (Seifert 2007). The three ant species differed in body size: *L. fuliginosus* 3.7±0.1 mm (N=6, mean ± SE), *M. ruginodis* 4.2±0.1 mm, *T. nylanderi* 1.9±0.04 mm (measured with a measuring eyepiece and a binocular to the nearest 0.1 mm).

Diaspores Diaspores of up to 16 plant species were used for cafeteria experiments and chemical analyses. Classification of diaspores as elaiosome-bearing or non-elaiosome-bearing followed empirical observations of diaspore structures with a binocular.

Eight species were classified as elaiosome-bearing diaspores: *Asarum europaeum* (Aristolochiaceae), *Chelidonium majus* (Papaveraceae), *Corydalis cava* (Fumariaceae), *Corydalis lutea* (Fumariaceae), *Hepatica nobilis* (Ranunculaceae), *Veronica hederifolia* (Scrophulariaceae), *Viola odorata* (Violaceae), and *Viola arvensis* (Violaceae). Another eight species have diaspores without elaiosomes: *Allium ursinum* (Alliaceae), *Anemone nemorosa* (Ranunculaceae), *Anemone ranunculoides* (Ranunculaceae), *Capsella bursa-pastoris* (Brassicaceae), *Eranthis hyemalis* (Ranunculaceae), *Helleborus viridis* (Ranunculaceae), *Ranunculus auricomus* (Ranunculaceae), and *Stellaria holostea* (Caryophyllaceae).

In order to calculate the approximate mean volume of each diaspore species, the diameter of 6 diaspores of each species was measured in top view position with a binocular and a measuring eyepiece (to the nearest 0.1 mm) and taken to estimate the volume by assuming a spherical.

Diaspores were collected in the vicinity of Würzburg in May and June 2008 and stored at –18 °C. Diaspores used for chemical analyses were treated in the microwave for one min at ca. 240 Watt, afterwards kept at 60 °C for 5–7 d, and stored in a desiccator. Diaspores used for biotests were taken out of the freezer prior the tests and defrosted at room-temperature.

Cafeteria Dual and Multiple Choice Tests

Cafeteria experiments were conducted in a deciduous forest in the Steigerwald, close to Fabrikschleichach (49°92′N, 10° 56′O) in northern Bavaria, Germany. Thus, habitats of field experiments did not match habitats of diaspore collection. A bait of sugar and tuna was placed near the ants' nest prior to the experiments to enhance the ants' interest. Depending of the ant species, baits were offered for 1–4 hr to ensure recruitment to the new food source. With the start of the experiments, baits were removed and replaced by a cafeteria, a petri-dish equipped with filter paper and with four openings in its rim to allow ant access. Ant response was observed for 60 min. Every 5 min the number of remaining items per species was recorded.

Dual Choice Tests To investigate the role of elaiosomes for the enhanced attractiveness of diaspores, diaspores of two haphazardly chosen plant species with large numbers of available diaspores of a suitable size for efficient dissection, *H. nobilis* and *V. odorata*, were dissected in elaiosomes and seeds. For each plant species, separated elaiosomes and seeds (12 of each) were offered simultaneously in the cafeteria to *L. fuliginosus* and *M. ruginodis* (= 24 items replicated three or four times at different colonies).

Multiple Choice Tests To investigate general preferences of ants, diaspores of all 16 plant species were simultaneously offered to colonies of *L. fuliginosus*, *M. ruginodis*, and *T. nylanderi* (five items per species=80 items per biotests, replicated four or five times at different colonies).

Seeds and Elaiosome Preparation for Extraction

Elaiosomes were dissected from seeds, and both were analyzed separately and compared with non-elaiosome-bearing seeds. For each sample, 10–20 seeds were ground in a swing mill (1 min, 30 swings per second; Retsch, Haan, Germany) and 5–7 mg were used for each analysis. Elaiosomes were weighed out in portions of 2–4 mg per analysis.

Fatty Acids Fatty acids of seeds and elaiosomes were qualitatively analyzed by gas chromatographic analyses. The plant material was extracted with 200 µl chloroform ($CHCl_3$) for 3–4 (elaiosomes) and 1–2 (seeds) min. Samples were dried under a nitrogen flow. In order to hydrolyze and convert lipids to methyl esters, samples were resolved in 1 ml methanol-HCl-mixture (c=8 ml HCl (37 %)/40 ml $CHCl_3$), and incubated for 1 h at 70 °C. Afterwards, the samples were dried again under a nitrogen flow, and resolved in 50 µl $CHCl_3$ and 20 µl BSTFA. Extracts were analyzed in a gas chromatograph (gradient: start temperature: 55 °C for 2 min, heat to 160 °C with 3 °C/min, 160–165 °C with 1 °C/min, 165–300 °C with 5 °C/min, 300 °C for 34 min) linked with a mass spectrometer (MS; Agilent Technologies, Boeblingen, Germany: GC-MS 6890N+5973 Network Mass Selective Detector).

Fatty acid methyl esters were identified by comparison of MS spectra and retention times to those of separately analyzed pure fatty acids (Sigma Aldrich) and to a commercial mass spectral data base (WILEY Registry of Mass Spectral Data).

Amino Acids Aquaeous extracts of seeds and elaiosomes were analyzed in an amino acid analyzer (Eppendorf-BIOTRONIC, Hamburg, Germany; PEEK-column) using liquid chromatography. Ninhydrin allows the photometric detection of amino acid products, detectable at 570 nm and 470 nm (for proline), respectively. Elaiosomes and ground seeds were extracted with 200 µl distilled water, centrifuged and the supernatants were separated from the pellet. Supernatants were cooked for 1–2 min, cooled to room temperature, and centrifuged again. Fifteen µl 12.5 % sulfosalicylic acid were added to 75 µl of each supernatant and incubated. Seventy-five µl of this supernatant were mixed with 75 µl sample dilution buffer and centrifuged. The pellet was used for analyses of protein-bound amino acids. Two hundred µl HCl (6 N) were added to the pellet and cooked for 4 hr. After the samples cooled to room temperature, they were centrifuged, the pellets discarded, and the supernatant cooked again. Cooking was repeated with 200 µl distilled

water. The subsequent procedure followed the protocol for the analysis of free amino acids, but 20 μl sample extract were blended with 80 μl sample dilution buffer. Quantification and identification of amino acid profiles of seeds and elaiosomes were achieved by the comparison of retention times with those of an external standard, which contained alanine, β-alanine, arginine, asparagic acid, asparagine, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine.

Sugars Aqueous extracts of ground seeds and of elaiosomes were analyzed via high performance liquid chromatography (HPLC; Dionex 4500i, Dionex, Idstein, Germany) with a pulsed electrochemical detector. Columns used were CarboPac PA1-columns (Dionex, Idstein, Germany). Eluation was isocratic with 0.08 M NaOH (pH=13). Elaiosome and ground seed samples were extracted with 5×1 ml or 2× 1 ml twice-distilled water, respectively. For each sample, supernatants of these 1 ml extracts were combined and cooked for 5 min. Fifty - 100 mg Serdolit-resin (Serva, Boehringer Bioproducts Partnership, Ingelheim, Germany) were added to 1 ml samples of these combined extracts. Six hundred μl supernatants of these were used for undiluted samples as well as 1:10-dilution samples. If both values fit in the reliable measurement range of the HPLC-unit, the average of both measurements was incorporated in further evaluations; otherwise the more reliable one was taken. Quantification and identification of sugar profiles of seeds and elaiosomes were achieved by the comparison of retention times with those of an external standard, which contained glucose, fructose, sucrose, arabinose, maltose, and mannitol (50 μl/1 l).

Statistical Analyses Transportation rates of each diaspore species observed in multiple choice tests were analyzed via a Kaplan-Meier-Estimation of survival (= no transport) of each diaspore species (pooled data for each combination of ant and plant species):

$$\widehat{S}(t_i) = \prod_{t(i)\le t} \left(1 - \frac{d_i}{n_i}\right)$$

with S(t) = the estimated probability of seeds not to be transported for any particular one of the t time periods, n_i = the number of diaspore at the beginning of time period t_i, and d_i = the number of diaspore that are transported during time period *ti.*

An exponential regression curve was fitted to the resulting graph of survival probability. The reciprocal of the negative logarithm of its slope provides the reference value for the attractiveness of the respective plant species. In order to get more descriptive values, these reference values were multiplied by ten and taken as preference indices (R, version 2.9.2, R-package Survival).

In order to relate chemical and structural diaspore properties to preferences, we performed *one-tailed Students t-tests* (presence of any kind of handle, presence of an elaiosome), *one-way ANOVA* (seed surface structures sorted in four categories: smooth, smooth + depressions, hirsute/hairy, wrinkled) or *one-tailed linear regressions* for univariate exploratory variables (seed volume, elaiosome/seed-size ratio, relative contents of saturated fatty acids, unsaturated fatty acids and poly-unsaturated fatty acids, C16, C18:1, and C18:2 fatty acids, total amino acids, free amino acids, essential free amino acids and proteins, contents of total sugars, monosaccharides, and disaccharides). Since only potentially attractive substances were examined, all correlations were one-tailed, disregarding negative relationships. Multivariate compositional similarities (composition of fatty acids, amino acids, and sugars) were tested with *Mantel tests*. In order to contrast the role of elaiosomes with non-elaiosome-bearing seeds for ants' preferences for diaspores, chemical data for elaiosomes were taken to represent the chemistry of the entire diaspores, disregarding the corresponding seeds in these analyses. *Mantel tests* based on *Pearsons correlations* were performed to relate Bray-Curtis similarities in chemical composition to differences between preference values.

The composition of fatty acids, amino acids, and sugars, respectively, of seeds, their corresponding elaiosomes and non-elaiosome-bearing seeds were analyzed as proportional data (i.e., the total of all compounds of each chemical class was 100 %). Non-metric multi-dimensional scaling (*meta-NMDS*) was used for graphical display. Compositional differences among groups were evaluated with permutational multivariate analyses of variance using distance matrices (*Adonis*) (both R-package Vegan, Bray-curtis distances of proportions), proportional contents among groups were compared with *ANOVA* followed by *Tukey HSD post hoc tests*, among pairs with *Students t-test*. Statistics were performed in R, version 2.9.2.

Results

Analyses of Diaspores Diaspores varied markedly in size, shape and surface structure (Fig. 1, Table 1). Three of the non-elaiosome-bearing diaspore species had grip-like handles. Elaiosomes varied species-specifically in shape and size, resulting in strong variation in elaiosome/seed-size ratios (Table 1).

Ant Preference Tests In dual choice cafeteria experiments, *M. ruginodis* clearly preferred isolated elaiosomes over

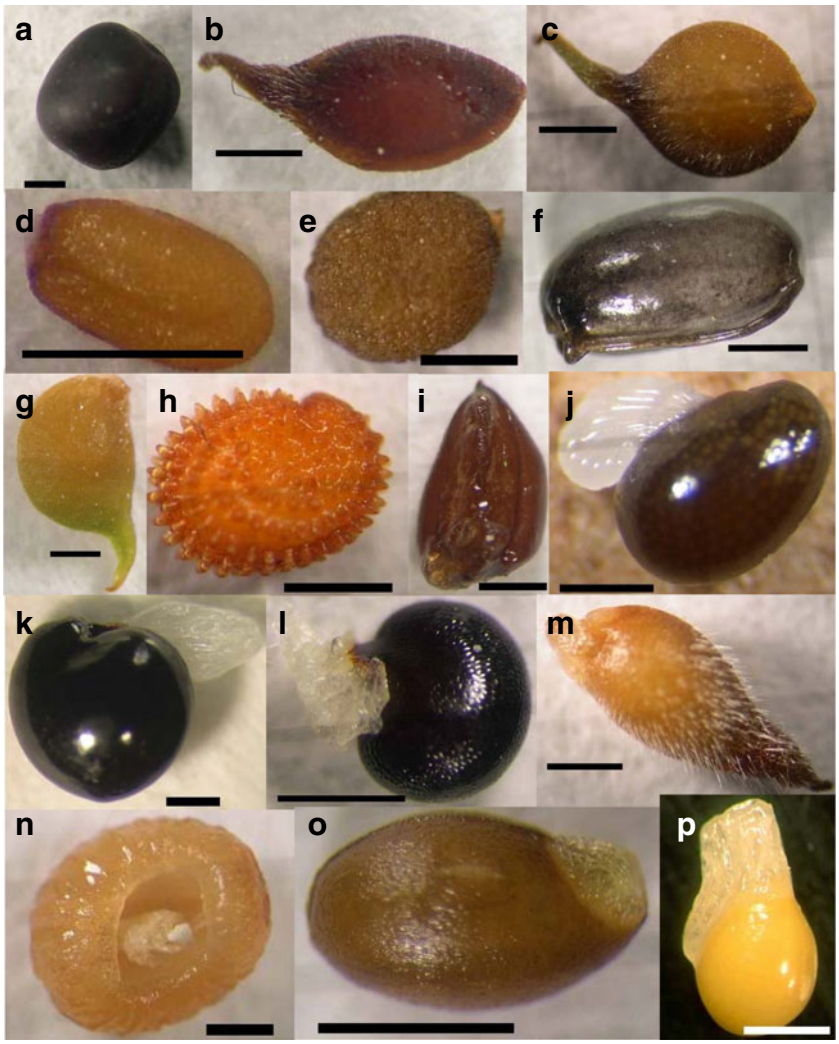


Fig. 1 Photographs of diaspores used for cafeteria experiments: a *Allium ursinum,* b *Anemone nemorosa*, c *Anemone ranunculoides,* d *Capsella bursa-pastoris,* e *Eranthis hyemalis,* f *Helleborus viridis, g Ranunculus auricomus*, h *Stellaria holostea*, i *Asarum europaeum,* j *Chelidonium majus,* k *Corydalis cava,* l *Corydalis lutea,* m *Hepatica nobilis,* n *Veronica hederifolia,* o *Viola odorata*, p *Viola arvensis*. Scale bars=1 mm

conspecific seeds detached from elaiosomes of both plant species (mean removal rates *V. odorata* after 30 min: elaiosomes 91.7±8.3 %, seeds 47.9±16.4 %; and *H. nobilis* after 30 min: elaiosomes 100±0 %, seeds 13.9±10 %). *Lasius fuliginosus* showed similar preferences for *V. odorata* (after 30 min: elaiosomes 97.2±2.8 %, seeds 2.8±2.8 %), whereas preferences were less pronounced for *H. nobilis* (after 30 min: elaiosomes 5.6±4.5 %, seeds 2.8±2.3 %).

Transportation rates of simultaneously offered diaspores of 16 plant species with and without elaiosomes (i.e., two groups) were converted into preference indices for each plant and each of the three ant species (Table 1). Diaspore choice preference indices correlated between *M. ruginodis* and *T. nylanderi* (*Linear regression* R^2=0.35, P=0.005) and *L. fuliginosus* and *M. ruginodis* (R^2=0.21, P=0.021), but not between *L. fuliginosus* and *T. nylanderi* (R^2=0.01, P=0.19).

Diaspore choice of *L. fuliginosus* and *M. ruginodis* correlated with the presence of any kind of handle attached to a seed. However, correlation of diaspore preferences with the presence of an elaiosome was even stronger (Table 2). Among elaiosome-bearing seeds, these ants' preferences were affected by elaiosome/seed-size ratio. Chemically, diaspore choice of *L. fuliginosus* and *M. ruginodis* correlated with the proportional amino acid composition, with contents of total free amino acids, and with contents of essential free amino acid. Moreover, preferences of *L. fuliginosus* corresponded with contents of oleic acid, whereas diaspore preferences of *M. ruginodis* correlated with contents of saturated fatty acids and palmitic acid (Table 2).

In contrast to *L. fuliginosus* and *M. ruginodis*, diaspore preferences of *T. nylanderi* did not correlate with structural diaspore properties, but were predicted by proportional amino acid compositions, and the contents of saturated fatty acids and palmitic acid (Table 2).

Fatty Acids Elaiosomes, their corresponding seeds and non-elaiosome-bearing seeds differed significantly in their fatty acid composition. Elaiosomes of different seed species were more similar to each other than each elaiosome and its corresponding seed. Non-elaiosome-bearing seeds were

Table 1 Volume, surface structure, shape and elaiosome/seed-size ratio of diaspores used for biotests and chemical analyses (mean ± SE, N=6) and preference indices of three ant species calculated from observed transport behavior in cafeteria field experiments

Plant species	Diaspore volume (mm^3)	Diaspore surface structure	Diaspore shape	E/S size ratio	Preference indices		
					Lasius fuliginosus	*Myrmica ruginodis*	*Temnothorax nylanderi*
Allium ursinum	14.6±0.6	smooth	globular	no elaiosome	1.6	2.1	0.3
Anemosa nemorosa	16.8±3.1	hirsute	plane-egg-shaped + handle	no elaiosome	1.4	2.0	0.3
Anemosa ranunculoides	11.7±1.1	hirsute	globular + handle	no elaiosome	1.7	1.8	0.3
Capsella bursa-pastoris	0.3±0.0	smooth, slightly wrinkled	egg-shaped	no elaiosome	0.3	0.3	0.3
Eranthis hyemalis	7.8±0.5	wrinkled	globular	no elaiosome	1.6	1.7	0.3
Helleborus viridis	31.9±3.3	smooth	cylindrical	no elaiosome	2.3	1.7	0.3
Ranunculus auricomus	16.1±1.0	hirsute	plane-egg-shaped + handle	no elaiosome	1.7	1.9	0.3
Stellaria holostea	7.2±1.1	many small conical mounds	globular- egg-shaped	no elaiosome	0.3	2.7	0.3
Asarum europaeum	15.5±1.1	smooth	half-globular, egg-shaped	0.47±0.0	3.9	2.9	0.3
Chelidonium majus	1.4±0.1	smooth	kidney-shaped	0.26±0.0	2.2	2.8	1.7
Corydalis cava	9.8±0.7	smooth	globular	0.47±0.0	2.5	2.6	0.3
Corydalis lutea	7.2±0.6	smooth, small depressions	globular	0.39±0.0	1.9	2.3	0.3
Hepatica nobilis	9.0±1.3	hairy	clubbed	0.06±0.0	2.0	2.1	0.3
Veronica hederifolia	12.3±0.8	smooth, clear depressions	egg-shaped	0.21±0.18	1.8	2.0	0.3
Viola arvensis	1.3±0.1	smooth, small depressions	egg-shaped	0.04±0.0	2.0	3.7	2.3
Viola odorata	9.9±0.5	smooth	egg-shaped	0.19±0.0	2.8	2.7	1.6

more heterogeneous in their fatty acid composition; they differed significantly from elaiosomes but not from elaiosome-bearing seeds (Fig. 2a, Tables 3 and 4; names and contents of fatty acids are listed in Supplemental Table 1).

Elaiosome-bearing seeds and elaiosome-less seeds were dominated by unsaturated fatty acids, mostly identified as linoleic acid (C18:2), and in smaller ratios oleic-acid (C18:1). Relative linoleic acid contents in both seed groups were about twice as high as in elaiosomes. In elaiosomes, relative contents of saturated fatty acids, dominated by palmitic acid, were significantly higher compared to elaiosome-bearing seeds and non-elaiosome-bearing seeds. Ratios of oleic acid (C:18-1), the predominant mono-unsaturated fatty acid, varied between the three groups, with lowest contents in non-elaiosome-bearing seeds and higher contents in elaiosomes compared to their corresponding seeds in five of seven species investigated (Table 3).

Amino Acids The proportional total amino acid composition revealed high similarities within elaiosomes, whereas their corresponding seeds and non-elaiosome-bearing seeds did not cluster as homogenous groups (Fig. 2b, Tables 3 and 4; for names and contents of amino acids see Supplemental Table 2).

Non-elaiosome-bearing seeds, elaiosome-bearing seeds, and elaiosomes had similar total amino acid contents (Table 3). However, in elaiosomes, relative free amino acid contents were about three times higher than those in their corresponding seeds (significant higher content in elaiosomes compared to their corresponding seeds were detected for alanine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine) and 3.7 times higher than in non-elaiosome-bearing seeds (significant higher content in elaiosomes compared to non-elaiosome-bearing seeds were detected for alanine, histidine, isoleucine, leucine, phenylalanine, proline, threonine, tyrosine, valine). Moreover, the relative free essential amino acid content in elaiosomes was 3.6 times higher than in their corresponding seeds (essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine) and four times higher than non-elaiosome-bearing seeds (Table 3). However, within the group of highly attractive elaiosome-bearing seeds, amino acid

Table 2 Structural and chemical diaspore data (presence of any kind of handle, presence of an elaiosome, elaiosome/seed-size ratios, diaspore volume, surface structure, proportional composition of amino acids, total free amino acid contents, essential free amino acids contents, proportional compositions of fatty acids, saturated fatty acid content, palmitic acid content (C16), oleic acid content (C18:1), proportional compositions of sugars) correlated with diaspore preference indices of three ant species. Only significant diaspore properties are listed for each species ($P<0.05$).

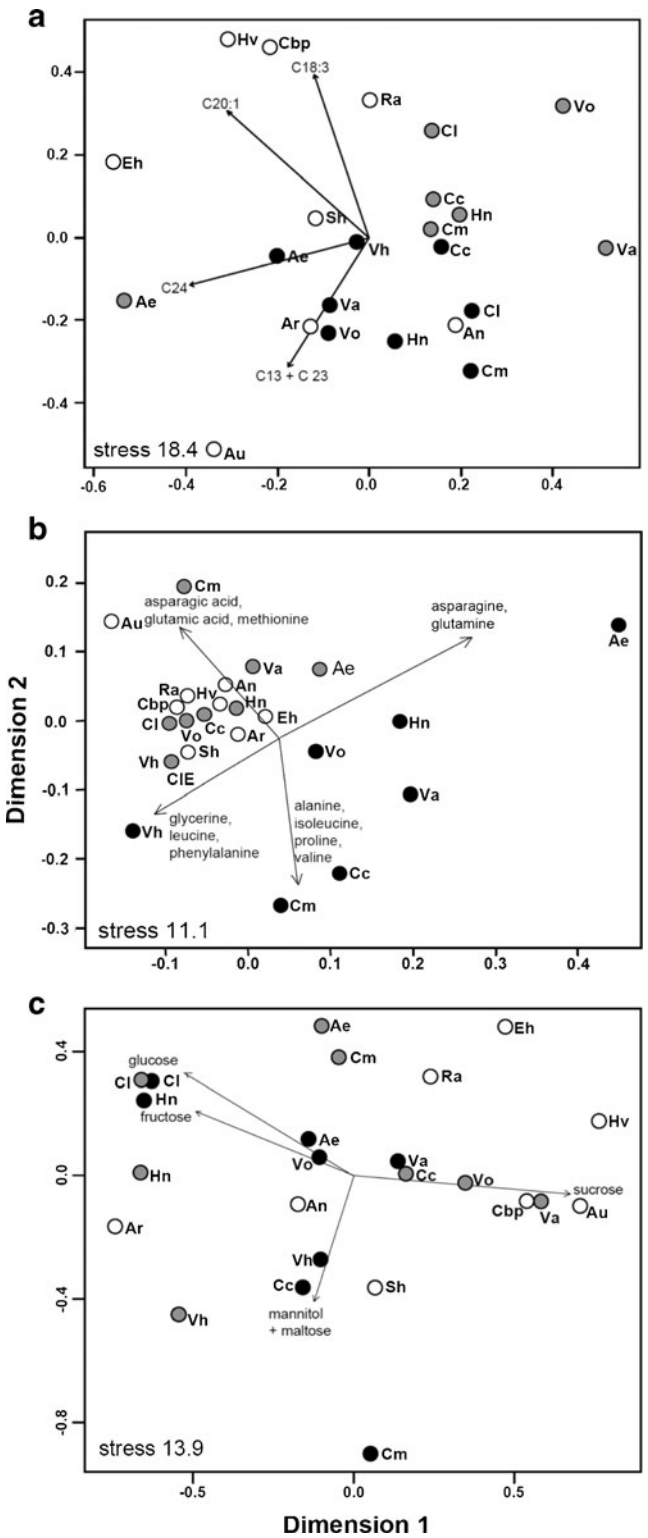
		P	
Lasius fuliginosus			
Presence of any kind of handle	$t=2.4$	0.017	*one-tailed Students t-test*
Presence of an elaiosome	$t=5.5$	< 0.001	*one-tailed Students t-test*
Elaiosome/seed-size ratios	$R^2=0.00$	0.005	*Linear regression*
Proportional amino acid composition	$R=0.28$	0.036	*Mantel test*
Total free amino acid contents	$R^2=0.47$	0.001	*Linear regression*
Essential free amino acids contents	$R^2=0.24$	0.015	*Linear regression*
Oleic acid content (C18:1)	$R^2=0.17$	0.036	*Linear regression*
Myrmica ruginodis			
Presence of any kind of handle	$t=2.1$	0.029	*one-tailed Students t-test*
Presence of an elaiosome	$t=7.5$	< 0.001	*one-tailed Students t-test*
Proportional amino acid composition	$R=0.32$	0.005	*Mantel test*
Total free amino acid contents	$R^2=0.20$	0.046	*Linear regression*
Essential free amino acids contents	$R^2=0.13$	0.045	*Linear regression*
Saturated fatty acid content	$R^2=0.56$	< 0.001	*Linear regression*
Palmitic acid content (C16)	$R^2=0.43$	0.005	*Linear regression*
Temnothorax nylanderi			
Proportional amino acid composition	$R=0.32$	0.05	*Mantel test*
Saturated fatty acid content	$R^2=0.45$	0.002	*Linear regression*
Palmitic acid content (C16)	$R^2=0.75$	< 0.001	*Linear regression*

profiles varied strongly with only three single amino acids supporting a homogenous pattern for the content and combination of amino acids. Compared to elaiosome-bearing seeds (ES) and non-elaiosome-bearing seeds (NES) separated elaiosomes (E) had significantly lower relative contents of asparagic acid (*Students t-test:* E vs. ES $t=2.42$, $P=0.030$, E vs. NES $t=2.78$, $P=0.015$) and glutamic acid (E vs. ES $t=2.56$, $P=0.023$, E vs. NES $t=5.69$, $P<0.001$) but significantly higher relative contents of tyrosine (*Students t-test:* E vs. ES $t=2.37$, $P=0.032$, E vs. NES $t=2.70$, $P=0.017$). Characteristic free amino acid profiles of seeds lacking elaiosomes and corresponding seeds were given by arginine, asparagine and glutamic acid (proportional contents all >10 %), whereas elaiosomes were characterised by asparagine, glutamine, proline and thyrosine (proportional contents all >10 %).

Sugars The proportional sugar compositions of elaiosomes, their corresponding seeds and other seeds showed no clear differences between the three groups (Fig. 2c, Tables 3 and 4; for names and contents of amino acids see Supplemental Table 3). The total sugar content was highest, but also most variable in non-elaiosome-bearing seeds (Table 3). Monosaccharide contents of elaiosomes and their corresponding seeds were approximately twice as high as those in non-elaiosome-bearing seeds, whereas the disaccharide content was twice as high in non-elaiosome-bearing seeds compared to the other groups (Table 3). However, none of these differences were statistically significant.

Discussion

Several studies have focused on the mechanisms that drive diaspore dispersal by ants, but most studies have examined strong simplified myrmecochorous systems with a single ant and only one or few plant species (e.g. Gorb and Gorb 1995, 1999b). These studies propose various mechanisms and signals that release seed transport behavior in ants. Extensive analyses of the chemical profile of a broader spectrum of diaspores were presented by Fischer et al. (2008), but were not combined with observations on the diaspore choice behavior of ants. In the present study, we classified diaspores of 16 plant species via empirical observations as elaiosome-bearing and non-elaiosome-bearing, and we offered both diaspore types simultaneously to three ant species in order to detect choice preferences. In accordance with various studies on myrmecochorous systems (e.g., Sernander 1906; Bresinsky 1963; Hughes and Westoby 1992; Peters et al. 2003), our cafeteria experiments confirmed that elaiosome-bearing seeds generally are more attractive for ants than seeds without elaiosomes. Among the most attractive diaspores that achieved strong transportation rates, mirrored in high preference indices, diaspores of two plant species were similarly preferred by all three ant species and quickly removed in high numbers, *Viola odorata* and *Chelidonium majus*, both with elaiosome-bearing seeds. Further diaspores species with high preference values were elaiosome-bearing *Asarum europaeum* and *Corydalis*



◀ **Fig. 2** *Non-metric multidimensional scaling (NMDS;* Bray-curtis distances of proportions*)* of the proportional composition of a) fatty acids b) amino acids and c) sugars of non-elaiosome-bearing seeds, elaiosome-bearing seeds and their corresponding elaiosomes. White circles = non-elaiosome-bearing seeds, grey circles = elaiosome-bearing seeds, black circles = elaiosomes. Abbreviations: Au = *Allium ursinum*, An = *Anemosa nemorosa*, Ar = *Anemosa ranunculoides*, Cbp = *Capsella bursa-pastoris*, Eh = *Eranthis hyemalis*, Hv = *Helleborus viridis*, Ra = *Ranunculus auricomus*, Sh = *Stellaria holostea*, Ae = *Asarum europaeum*, Cm = *Chelidonium majus*, Cc = *Corydalis cava*, Cl = *Corydalis lutea*, Hn = *Hepatica nobilis*, Vh = *Veronica hederifolia*, Va = *Viola arvensis*, Vo = *Viola odorata*

cava diaspores preferably transported by *Myrmica ruginodis* and *Lasius fuliginosus*, but rejected by *Temnothorax nylanderi*, and elaiosome-bearing *Viola arvensis* diaspores frequently transported by *M. ruginodis* and *T. nylanderi.*

Observation that some elaiosome-bearing seed species are well accepted by only two of the three ant species tested may simply reflect differences in body size. *Temnothorax. nylanderi* is approximately only half as large as *M. ruginodis* and *L. fuliginosus* and not able to carry larger diaspores, independently from the ants' preferences. *Temnothorax nylanderi* were regularly observed to feed on elaiosomes of such large diaspores (C. Becker pers. obs.). However, for both larger ant species, *M. ruginodis* and *L. fuliginosus,* elaiosome/seed-size ratios were of minor importance. Both species are similarly attracted to *A. europeaum*, *C. cava,* and *V. odorata*, although elaiosome/seed-size ratios of these three species varied strongly, whereas the high elaiosome/seed size ratio of *C. lutea* did not noticeably stimulate the ants' interest.

Thick and fleshy elaiosomes attached to seeds may simply represent grips that facilitate the transport by ants (Gorb and Gorb 1999a) or attract ants with high levels of (essential) nutrients (Fischer et al. 2008). Despite remarkable proportional variations within elaiosome-species, our analyses of free fatty acids revealed comparably low contents of poly-unsaturated linoleic acid, medium levels of mono-unsaturated oleic acid, and relatively high proportions of saturated fatty acids in highly attractive elaiosomes, with palmitic acid corresponding to seed attraction for *M. ruginodis* and *T. nylanderi.* However, insects are able to synthesize saturated and also mono-unsaturated fatty acids, but not polyunsaturated fatty acids (Dadd 1973). Due to such low contents of polyunsaturated fatty acids in elaiosomes, it seems rather improbable that lipid requirements drive the ants' seed choice. However, since we did not differentiate between free and bounded fatty acids, we cannot entirely exclude any effects of fatty acids on ant-seed preferences.

Differences in sugar profiles of elaiosomes, elaiosome-bearing seeds, or elaiosome-less seeds did not correspond with diaspore preferences of the three ant species, although high proportional contents of metabolically convenient and easy digestible monosaccharides could generally be involved in insect attraction. Therefore, the collection of elaiosome-bearing seeds seems not to refer to the ants' requirements for fatty acids or sugars.

However, preferences of all three ant species were well predicted by the amino acid composition in diaspores. Elaiosomes generally contained larger amounts of free essential and other free amino acids than their corresponding seeds

Table 3 Proportional composition of fatty acids (saturated, unsaturated, poly-unsaturated, linoleic acid (C18:2), oleic acid (C18:1), and palmitic acid (C16)), amino acids (protein, total free and free essential) and sugars (monosaccharides and disaccharides) and total contents of amino acids and sugars in a) non-elaiosome-bearings seeds, b) elaiosome-bearing seeds and c) elaiosomes of 16 plant species. nd = no data

		Fatty acids (%)						Amino acids				Sugars		
		Saturated	Palmitic acid (C16)	Mono-unsaturated	Oleic (C18:1)	Poly-unsaturated	Linoleic acid (C18:2)	Total (mg/g DW)	Proteins (%)	Total free (%)	Free essential (%)	Total (mg/g DW)	Monosaccharides (%)	Disaccharides (%)
a) non-elaiosome-bearing seeds (NES)	*Allium ursinum*	18.7	13.7	30.6	30.4	50.7	50.7	68.3	99.8	0.2	0.1	6.9	5.32	94.7
	Anemosa nemorosa	10.9	7.7	13.3	13.3	75.8	75.8	73.8	89.8	10.2	3.4	15.0	65.0	35.0
	Anemosa ranunculoides	14.2	9.5	18.0	17.8	67.8	67.8	61.5	78.5	21.5	4.5	15.7	72.9	27.1
	Capsella bursa-pastoris	16.7	7.4	22.1	13.3	61.2	27.0	111.3	97.4	2.6	0.8	20.0	9.5	90.5
	Eranthis hyemalis	21.9	14.4	32.1	18.4	46.1	33.9	41.7	86.7	13.3	1.3	26.7	45.3	54.7
	Helleborus viridis	11.3	8.8	13.1	6.1	75.6	50.3	99.3	94.2	5.8	0.8	45.9	6.0	94.0
	Ranunculus auricomus	14.2	8.5	17.2	16.6	68.5	65.8	90.1	96.6	3.4	0.4	9.8	38.1	61.9
	Stellaria holostea	29.5	20.1	25.1	24.8	45.4	39.1	57.6	92.1	7.9	1.2	27.2	47.8	52.2
	mean ± SE (*N*=8)	**17.2**±2.2	**11.3**±1.6	**21.5**±.6	**17.6**±2.6	**61.4**±4.4	**51.3**±6.2	**75.5**±8.2	**91.9**±2.4	**8.1**±2.4	**1.6**±0.6	**20.9**±4.4	**36.3**±9.4	**63.8**±9.4
b) elaiosome-bearing seeds (ES)	*Asarum europaeum*	9.6	6.3	58.5	13.3	31.8	31.8	89.0	73.4	26.6	3.4	6.5	91.7	8.3
	Corydalis cava	16.3	11.9	26.0	22.0	57.7	57.7	70.9	71.4	5.5	0.8	11.9	35.1	64.9
	Corydalis lutea	10.5	8.8	6.3	6.0	83.2	83.2	59.2	98.1	1.9	0.6	1.9	100	0
	Chelidonium majus	6.11	3.5	16.0	16.0	77.9	77.9	99.4	97.7	2.3	0.6	6.7	78.7	21.3
	Hepatica nobilis	11.6	7.5	22.3	22.3	66.2	66.2	53.5	82.7	17.3	3.6	18.8	91.4	8.7
	Veronica hederifolia	14.5	8.5	69.7	69.4	15.9	15.9	29.2	95.1	3.9	1.2	25.1	100	0
	Viola arvensis	13.0	9.4	18.6	18.3	68.4	68.4	50.5	86.4	13.7	2.2	21.1	9.0	91.0
	Viola odorata	12.8	9.1	22.7	22.4	64.5	64.5	60.4	91.6	8.4	1.5	10.3	21.3	78.7
	mean ± SE (*N*=8)	**11.8**±1.1	**8.1**±0.9	**30.0**±7.8	**29.2**±7.8	**58.2**±8.1	**58.2**±8.1	**64.0**±7.9	**90.1**±3.1	**9.9**±3.1	**1.7**±0.4	**12.8**±2.9	**65. 9**±13.4	**34.1**±13.4
c) elaiosomes (E)	*Asarum europaeum*	54.6	5.8	32.1	32.1	13.3	13.3	132.0	60.6	39.4	5.3	3.1	76.9	23.1
	Corydalis cava	25.3	14.3	34.6	32.1	40.1	35.4	106.9	53.6	46.4	12.1	5.1	82.8	17.3
	Corydalis lutea	29.8	27.6	24.8	21.5	45.4	29.0	73.0	95.0	5.0	1.2	6.2	100	0
	Chelidonium majus	37.9	34.8	24.9	24.5	37.2	37.2	88.4	62.8	37.2	10.1	2.5	20.4	79.7
	Hepatica nobilis	36.7	25.1	35.7	32.5	27.6	27.6	58.4	62.1	34.2	4.3	9.7	100	0
	Veronica hederifolia	nd	nd	nd	nd	nd	nd	34.2	77.7	17.0	5.7	19.2	71.6	28.4

Table 3 (continued)

		Fatty acids (%)						Amino acids			Sugars			
		Saturated	Palmitic acid (C16)	Mono-unsaturated	Oleic (C18:1)	Poly-unsaturated	Linoleic acid (C18:2)	Total (mg/g DW)	Proteins (%)	Total free (%)	Free essential (%)	Total (mg/g DW)	Monosaccharides (%)	Disaccharides (%)
	Viola arvensis	74.9	60.5	19.6	19.6	5.5	5.5	57.8	64.1	35.9	6.0	11.9	38.8	61.2
	Viola odorata	37.9	32.7	49.7	29.4	12.4	12.4	64.4	76.3	23.7	5.1	15.0	69.1	30.9
	mean ± SE (*N*=8)	**42.5**±6.4	**28.7**±6.6	**31.6**±3.8	**27.4**±2.1	**25.9**±5.9	**22.9**±4.7	**76.9**±11.0	**70.3**±4.8	**29.7**±5.3	**6.2**±1.2	**9.1**±2.1	**70.0**±9.9	**30.1**±9.9
ANOVA	*F*	**19.0**	**9.0**	1.1	1.6	**8.9**	**7.6**	0.6	**11.3**	**11.3**	**10.9**	3.4	2.8	2.8
	P	**<0.001**	**0.002**	0.37	0.23	**0.002**	**0.004**	0.56	**<0.001**	**<0.001**	**<0.001**	0.05	0.08	0.08
Tukey HSD post hoc	E - ES	**<0.001**	**0.002**			**0.006**	**0.004**		**0.002**	**0.002**	**0.002**			
	E - NES	**<0.001**	**0.008**			**0.003**	**0.019**		**0.001**	**0.001**	**0.001**			
	ES - NES	0.55	0.81			0.93	0.74		0.94	0.94	0.94			

ANOVA F- and *P*-values are given for statistical comparisons of chemical contents of non-elaiosome-bearings seeds, elaiosome-bearing seeds and elaiosomes across all species (significant *F*- and *P*-values in bold).

Table 4 Comparisons of proportional chemical compositions between elaiosomes, their corresponding seeds and non-elaiosome-bearing seeds (permutational multivariate analyses of variance based on distance matrices, *Adonis*)

	F	R^2	*P*
Fatty acids (saturated + unsaturated + poly-unsaturated)	3.2	0.24	0.006
elaiosomes vs. corresponding seeds	7.5	0.37	< 0.001
elaiosomes vs. non-elaiosome bearing seeds	7.0	0.35	< 0.001
elaiosome-bearing seeds vs. non-elaiosome-bearing seeds	1.9	0.12	0.12
Amino acids (soluble + proteins)	4.1	0.28	< 0.001
elaiosomes vs. corresponding seeds	4.2	0.23	< 0.001
elaiosomes vs. non-elaiosome bearing seeds	4.1	0.23	0.002
elaiosome-bearing seeds vs. non-elaiosome-bearing seeds	1.1	0.07	0.38
Sugars (mono- + disaccharides)	2.8	0.2	0.09

and non-elaiosome-bearing seeds, and therefore, can be regarded as highly nutritive. Proportions of free amino acids clearly corresponded with preference indices, at least for *L. fuliginosus* and *M. ruginodis*, although contents varied even within the most attractive diaspores. Low contents of asparagic and glutamic acid and higher proportions of asparagine, gluatmine, proline and tyrosine may positively affect the attraction of diaspores for ants. Amino acids also play a crucial role in attracting ants to extra floral nectaries (Lanza and Krauss 1984; Blüthgen and Fiedler 2004) by essentially contributing to the nectars taste (González-Teuber and Heil 2009) and by enhancing the nutritional value of extra floral nectar as food source (e.g., Baker and Baker 1973; Lanza and Krauss 1984; Blüthgen and Fiedler 2004). Similarly, elaiosomes may serve primarily as valuable, easily accessible nitrogen source in seasons of a lack of insect prey in early spring. Nitrogen limits growth, development, and fecundity of insects (Hagen et al. 1984) and, therefore, plays an important role in larval diets. Elaiosomes are preferentially fed to larvae (Fischer et al. 2005; Mayer et al. 2005), and colonies fed with elaiosomes produced about 100 % more larvae, with about 50 % higher larval weights than colonies fed with non-elaiosome containing food (Gammans et al. 2005; Fokuhl et al. 2007). Therefore, differences in the attractiveness of different plant species for the three ant species may refer to variation in demands for amino acids that probably depend on the condition of the colony. Ant foragers may adjust their food intake, e.g., the amount and balance of nutrients in response to the number of larvae and the need for certain compounds of workers (Dussutour and Simpson 2009). Differences in dietary choices of ants also may reflect variation in different life history traits (Blüthgen and Feldhaar 2010).

Ants obviously choose among seeds for those that are nutritionally most attractive, but may also perceive information on the suitability of seeds at the initial contact with the elaiosomes due to chemical cues. Former studies have identified oleic acid as the predominant fatty acid in elaiosomes, and have proposed oleic acid (e.g., Boulay et al. 2006; Pfeiffer et al. 2010) or its dimer diolein (e.g., Marshall et al. 1979; Skidmore and Heithaus 1988; Brew et al. 1989) or trimer tiolein (e.g., Fischer et al. 2008) releasing diaspore collecting behavior of ants. Oleic acid detected in our analyses may originally, entirely, or partly, occur in bound condition in diaspores, namely as diglycerides (diolein) or triglycerides (triolein). However, relative oleic acid contents did not correspond to the ants' preferences: they were not consistently increased in the most attractive diaspore species. Contents of oleic acid generally were high in elaiosomes, but similar to those in their corresponding seeds. Moreover, oleic acid proportions of some unattractive diaspores (e.g., *Eranthis hyemalis*, 18.4 %) were similarly high as highly preferred diaspores (e.g., *Viola arvensis*, 19.6 %.) Therefore, at least for the ant and plant species covered in the present study, it is unlikely that oleic acid or its dimer diolein exclusively or predominantly acts as a trigger substance, as claimed by several authors. In bioassays with seed-dummies prepared with elaiosome-extract fractions of different polarities, *M. ruginodis* strongly responded to polar fractions, which may contain amino acids and other polar compounds, but not to pure oleic acid or non-polar fractions that contain high levels of non-polar fatty acids (K. Reifenrath, unpublished data). These observations support Brew et al. (1989) who also suggested that not one single specific compound (e.g., 1,2-diolein) or class of compounds (e.g., all oleyl-containing lipids) in elaiosome-bearing seeds induces seed carrying behavior in Australian ant species, but that specific compositions of polar lipids may act as chemical cues as well as certain amino acids.

Although the identity of potential trigger compounds remains unclear, elaiosomes obviously enhance the attractiveness of diaspores. However, due to the fact that, at least for some plant species, non-elaiosome-bearing seeds have achieved similar preference values to elaiosome-bearing seeds, e.g., seeds of *S. holostea* for *M. ruginodis* and seeds of *H. viridis* for *L. fuliginosus*, nutritional rewards also may characterize the coats of non-elaiosome-bearing seeds. These contrasting findings may refer simply to an ambiguous classification of diaspores with and without elaiosomes, due to the high variability in structure, morphology, and anatomy of elaiosomes (Sernander 1906; Bresinsky 1963) and to their multiple evolutionary and developmental origins (Lengyel et al. 2010). Indeed, at least for some diaspores that lack elaiosomes, feeding marks were visible on the outer diaspore surface (C. Becker pers. observation), but it remains unclear if such partly fed diaspores keep the ability to germinate by ants. Detachment of elaiosomes does not generally affect the germination capacity (Mayer et al. 2005).

A recent review suggests relatively low costs for the production of elaiosomes, because of their wide-spread presence in various plant species and their multiple evolutionary developmental origins (Lengyel et al. 2010). Nevertheless, a cheap investment in elaiosomes may strongly affect the success of plants to disperse via rewarding ants as diaspore dispersers with easy accessible food supply. A generalistic dispersal by different ant species helps to reach a broad range of new habitats, which may differ in suitability for germination and establishment, but enhance the overall dispersal success. Being dispersed by one ant species or another may have divergent consequences, such as a variable dispersal distances, variable predation risks, and variable places of diaspore deposition subsequent to transportation.

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Defensive Spiroketals from *Asceles glaber* (Phasmatodea): Absolute Configuration and Effects on Ants and Mosquitoes

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Abstract Insects are the largest and most diverse group of organisms on earth, with over 1,000,000 species identified to date. Stick insects ("walkingsticks" or "phasmids", Order Phasmatodea) are known for and name-derived from their camouflage that acts as a primary line of defense from predation. However, many species also possess a potent chemical defense spray. Recently we discovered that the spray of *Asceles glaber* contains spiroketals [a confirmed major component: (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro[5.5]undecane, and a tentatively identified minor component: 2-ethyl-1,6-dioxaspiro[4.5]decane] and glucose. In this paper, we: 1) illustrate the identification of spiroketals and glucose in the defense spray of *A. glaber* by using Nuclear Magnetic Resonance (NMR), Gas Chromatography/Mass Spectrometry (GC/MS), and comparison with a synthetic reference sample; 2) provide the elucidation of the absolute configuration of the major spiroketal in that defense spray; and 3) demonstrate the effect of this compound and its enantiomer on both fire ants (*Solenopsis invicta*) and mosquitoes (*Aedes aegypti*).

Keywords Spiroketal · Phasmatodea · *Asceles glaber* · *Solenopsis invicta* · *Aedes aegypti* · Defense · Phasmatodea.

Introduction

Insects are known for their utilization of chemistry in communication and defense (Blum, 1981; Eisner et al., 2005; Laurent et al., 2005; Dossey, 2010). Stick and leaf insects (walkingstick insects, or phasmids; Order Phasmatodea), a relatively small order of insects composed of around 3,000 named species, are best known for their elaborate use of camouflage as a defense against predators (Bedford, 1978; Brock, 1999, 2009). However, many species produce a

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chemical spray from a pair of tegumental glands in their prothorax when disturbed (Scudder, 1876; Bedford, 1978; Dossey, 2010, 2011) (and references therein). The chemical composition of defensive sprays from only a few species has been analyzed (Schneider, 1934; Meinwald et al., 1962; Smith et al., 1979; Chow and Lin, 1986; Ho and Chow, 1993; Bouchard et al., 1997; Eisner et al., 1997; Schmeda-Hirschmann, 2006; Dossey et al., 2006, 2007, 2008, 2009; Prescott et al., 2009; Dossey, 2010, 2011). Besides the various secondary metabolites, glucose has been reported in the defensive sprays of *A. buprestoides* (Dossey et al., 2006), *P. schultei* (Dossey et al., 2006), *P. mocquerysi* (Dossey et al., 2007), *P. westwoodii* (Dossey et al., 2009), and *M. nigrosulfurea* (Prescott et al., 2009).

Asceles glaber (Günther, 1938) (Fig. 1) is a species of phasmid in the Suborder Euphasmatodea, Subfamily Necrosciinae. This is the most diverse Subfamily within the Stick and leaf insects, containing over 600 named species distributed throughout Asian and Australasian tropical forests. The natural history of these phasmids is poorly understood, but most members are winged, appear to be associated with the forest canopy, and exhibit specialized feeding habits (Bragg, 2001). *Asceles glaber* occurs in Myanmar and Thailand, and like many other phasmid species produces a liquid defense spray from a pair of tegumental glands in the anterior portion of their prothorax at minimum disturbance (Fig. 1b).

Spiroketals, sometimes referred to as spiroacetals, make up a large and diverse group of natural products that have been extensively reviewed in the literature (Booth et al., 2009). These have been isolated from a number of insect species (Tengö et al., 1982; Moore et al., 1994; Francke and Kitching, 2001; Goubault et al., 2008; Schwartz et al., 2008; Booth et al., 2009). However, to date, no spiroketals have been reported from stick insects. The first insect spiroketal was chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane) isolated from the European spruce bark beetle (*Pityogenes chalcographus*) (Francke et al., 1977; Booth et al., 2009). Various spiroketals possess important biological activities such as pheromone response (Francke et al., 1977; Weston et al., 1997) and chemical defense (Dettner et al., 1992; Zhang et al., 1999) in insects.

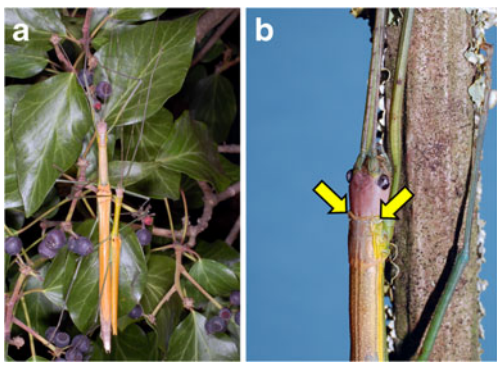

Fig. 1 Photographs of **a** an adult mating pair and **b** a close-up of head and prothorax of an adult female of *Asceles glaber* with arrows showing the position of the openings of its defensive glands. Photographs by Marco Gottardo

Here, we report the identification of two spiroketals (a confirmed major component (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane **1** and a tentatively identified minor component 2-ethyl-1,6-dioxaspiro[4.5]decane **2**) (Fig. 2) and glucose from the secretion of the prothoracic exocrine glands of both sexes of *A. glaber*. This paper 1) illustrates the identification of spiroketals and glucose in the defense spray of *A. glaber* by using Nuclear Magnetic Resonance (NMR), Gas Chromatography/Mass Spectrometry (GC/MS), and comparison with a synthetic reference sample, 2) provides the elucidation of the absolute configuration of the major spiroketal in that defense spray, and 3) demonstrates the effect of both enantiomers of the major *A. glaber* defensive spiroketal on both fire ants (*Solenopsis invicta*) and mosquitoes (*Aedes aegypti*).

Methods and Materials

General Experimental Procedures NMR experiments were performed with a 600 MHz 5-mm triple resonance cryogenic probe Bruker Biospin. Each sample was loaded into a 2.5-mm NMR tube (Norell, Inc.). During NMR experiments, the tube was held in a standard 10-mm spinner using a Bruker MATCH™ device, and the tube-MATCH-spinner combination was lowered vertically into the magnet on an air-column as usual. Sample temperature was regulated at 29°C. The spectrometer used for all NMR experiments was a Bruker Avance II 600. Additional NMR data acquisition parameters are found in the Supplemental Material with their respective spectra. All data acquisition, processing, and analysis were done with Bruker TopSpin© 2.0 software. Chemical shift assignments were made by referencing the resonances of the solvent proton impurity (benzene-d_5) to 7.16 for ^{1}H and 128.39 for ^{13}C, respectively. For spectra of samples in D_2O, chemical shift referencing was achieved by setting the anomeric ^{1}H to 5.22 and ^{13}C to 94.8 ppm of alpha glucose based on

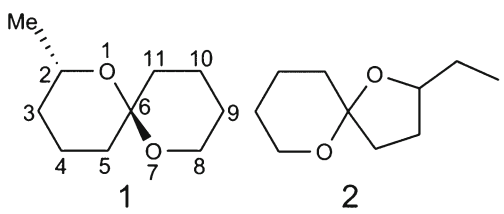

Fig. 2 Spiroketals identified in the defensive spray of the stick insect *Asceles glaber*: Major component (1) (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane (by NMR, GC/MS and comparison with an authentic synthetic standard) and a tentatively identified minor component (**2**) 2-ethyl-1,6-dioxaspiro[4.5]decane (by GC/MS)

the reported values for these resonances in the BMRB Metabolomic database (http://www.bmrb.wisc.edu/metabolomics/) (Ulrich et al., 2008).

GC/MS analyses were conducted on a TraceGC Ultra DSQ mass spectrometer (Thermo Scientific) equipped with an AT-5 ms column from Alltech (60 m×0.25 μm i.d.× 0.25 μm d_f). The injector was maintained at 280 °C, while the transfer line was set at 250 °C. The ion source was set at 180 °C and operated in electron impact (EI) mode, while He flow rate was at 1 ml/min. Two microliters (2 μl) of each sample were injected into the column in splitless mode at 40 °C for 2 min. The GC oven temperature was increased to 280 °C at a rate of 20 °C/min.

Asceles glaber Rearing and Sample Collection *Asceles glaber* specimens were obtained from descendence of females originally collected in the neighborhoods of Salok, Thailand, in 2003. For the present study, several males and females were reared in the laboratory at room temperature, moderate humidity conditions, and a 12:12 hrL/D photoperiod. Insects were kept in ventilated cages, and fed daily with *Hypericum* spp. leaves. Four independent samples, with volumes of approximately 1–50 μl, of defensive glandular secretion was obtained from a total of 51 milkings of *A. glaber*. Specifically, the following nomenclature will be used to refer to specific samples throughout this paper: *Asceles* 1 (26 milkings from 6 males to 4 females collected between December 2007 and Jenuary 2008), *Asceles* 2 (18 milkings from 3 males to 3 females collected between August 2008 and January 2009), *Asceles* 3 (5 milkings from 3 females collected between February and March 2008), and *Asceles* 4 (2 milkings from 1 female collected between February and March 2008). Sample *Asceles* 2 was larger despite the fewer number of milkings, because it contained the sprays from a large female that produced a large amount of secretion at each milking compared to other female specimens. It is also possible that *Asceles* 1 was quite small because sometimes not all the secretion was sprayed into the vial. For NMR, 1–5 μl of each milking was utilized (see captions of specific NMR figures) and either dissolved in D_2O or extracted with benzene-d_6. For GC/MS, approximately 1 μl of each sample was extracted with 0.5 ml of CH_2Cl_2 for analysis.

Spiroketals The two enantiomers of spiroketal **1** were synthesized as described in Whitaker (2012). Details of the synthesis will be published elsewhere.

(2S,6R)-(−)(E)- and (2R,6R)-(+)(Z)-2-methyl-1,7-dioxaspiro[5.5]undecane (1) Colorless oil; Optical rotations: $[\alpha]_D^{25}$=−62.02 (c 0.99, CH_2Cl_2), $[\alpha]_D^{25}$=+66.72 (c 1.06, CH_2Cl_2); ^{1}H NMR (600 MHz, C_6D_6) δ 3.75 (1H, m), 3.64 (1H, m), 3.54 (1H, m), 2.05 – 1.96 (2H, m), 1.66 – 1.08 (10H, m), 1.15 (3H, d, *J*=6.0 Hz) ppm; ^{13}C NMR (150 MHz, $CDCl_3$) δ 95.6, 65.1, 60.1, 36.2, 35.6, 33.2, 25.7, 22.2, 19.6, 19.1 ppm; The synthetic material was spectroscopically consistent with reported literature (Ghosh et al., 2006).

Quantification of Spiroketal 1 GC/MS was utilized for quantification of spiroketal **1** in each of the *A. glaber* spray samples collected. For these experiments, 1 μl of each sample was dissolved in 500 μl of dichloromethane (DCM) and was then analyzed by GC/MS. Based on preliminary results, *Asceles* 1 and 2 were diluted 3 and 10 fold, respectively, for the quantification. For the external calibration, synthetic spiroketal compound 1, equivalent to 1.25 to 12.5 μg/μl of sample, was prepared fresh. All solutions were injected at least three times in a 24-hr period. The method was validated by injecting the original *Asceles* 2 solution and a day-old standard solution at the end of the work list. Blank analysis consisted of solvent injections using the DCM that was used for the dilutions.

Absolute Configuration of Spiroketal 1 Determination GC/MS using an enantiomer selective column was utilized to determine the absolute configuration of the primary spiroketal component in *A. glaber* defense spray. GC/MS analyses were conducted on the same TraceGC Ultra DSQ mass spectrometer (Thermo Scientific) but using a Beta DEX 120 column from Supelco (30 m×0.25 μm i.d.×0.25 μm d_f). The injector and transfer line were maintained at 200 °C while the EI ion source was set at 180 °C. High purity He was used as carrier gas at a flow rate of 1 ml/min. Four microliters (4 μl) of the same sample solutions used for the quantification were injected into the column in splitless mode at 40 °C for 2 min. The GC oven temperature was increased to 220 °C at a rate of 20 °C/min. The 2(*R*)-(+) and 2(*S*)-(−) synthetic standards were prepared in DCM as 100 μg/ml solutions and analyzed in the same manner using 0.5 μl injections.

Bioassays on Fire Ants

Olfactometer Bioassay The bioassay is similar to that described by Vander Meer et al. (1988). The volatile spiroketal was dissolved in pentane to make an initial test concentration of 1 %. The solvent control was pentane. Each treatment and control (10 μl) was applied to filter paper pieces (Whatman #1; 1×0.3 cm). Filter paper pieces containing the treatment and control were placed inside the entrances of each of the two arms of the Y-tube olfactometer connected to the airflow. Purified compressed air was passed through each of the two sample Y-tube arms at a rate of 100 ml/min (200 ml/min combined). The main body of the olfactometer was 12 cm long×1.5 cm id. Approximately 100 worker ants were placed in a piece of tygon tubing (7 cm long×1.0 cm id) closed with a wire mesh cap at the distal end. The other

end of the tubing was connected to the downwind arm of the Y-tube olfactometer. Worker ants walked to the bifurcation choice point and went to treatment or control. After 20 ants had made a choice, the apparatus was cleaned and returned to the previous position. Treatment and control samples were prepared again, and their positions in the Y-tube arms were reversed. The choice of another 20 workers ants was recorded, and the sum of the two results constituted one replicate (N=40). Due to the volatility of the test compounds, the experiments were terminated after 3 min. The experiment was replicated a minimum of 3 times. The *S. invicta* queen attractant found in the poison sac was used as a positive standard (0.33 queen poison sac equivalents per 1.5 μl hexane) (Vander Meer et al., 1980) to test proper function of the olfactometer. Result significance was measured by *chi-square* analysis with a null hypothesis of equal numbers of ants in each arm. Results where <35 % of the ants chose the treatment indicate significant repellency; whereas results of >65 % represents significant attraction to the treatment. Results between 35 and 65 % indicate neutral activity.

Contact Repellent Bioassay The test tray was comprised of a porcelain pan measuring 180×290×50 mm. The upper 3 mm of the pan was coated with Fluon© to preclude ants from escaping. A Petri dish nest cell (55 mm diam.) was placed at one end of the pan. The Petri dish had a 5 mm layer of Castone© dental cement on the bottom that acted as a moisture reservoir. The lid had a hole placed in the center to allow ant access. To protect the bottom of the pan from contamination by the test materials, 2.5 cm sq. pieces of aluminum foil were placed in the opposite end of the pan from the nest cell at each corner approximately 3.0 cm from the sides of the pan. No food or water was available to the ants during the bioassay. For the execution of the bioassay fifty micro liters of test material were introduced into the test chamber on a 2.0 cm sq. piece of Whatman© silicone treated filter paper (cat. # 2200 125) and was randomly assigned and placed on one of the Aluminum foil squares. The other aluminum square received a filter paper square with 50 μl of pentane as control. Placed on top of each filter paper square was a small wad of cotton soaked in 10 % sucrose to serve as a phagostimulant. Once test materials were in place, approximately 1 g of ants (starved for a minimum of 24 hr) was placed in the nest cell and a stopwatch was started. The number of ants actively feeding on the treatment and control cotton balls was recorded at 1-min intervals for a total of 5 min. Each experiment was replicated three times, each with a unique monogyne colony and in a different test tray.

Bioassays on Mosquitoes Aedes aegypti Bioassays on Mosquitoes (*Aedes aegypti*) were received from the colony maintained at the United States Department of Agriculture-Agricultural Research Service in Gainesville, FL, at the same location as the assays were conducted. Mosquitoes were maintained in the laboratory on water and a solution of 10 % sucrose in water. The laboratory photoperiod was 12:12 L:D. A draw box (Posey and Schreck, 1981) was used to select the appropriate number of female mosquitoes for the assays of repellency, attraction, and attraction-inhibition. Ages of mosquitoes selected for tests ranged from 5 to 12 d old.

Contact Repellency Repellency was assessed by using a cage test (Barnard et al., 2007), and treatments were tested with the "cloth patch" assay method described in depth previously (Katritzky et al., 2010). Treatments consisted of 2(*R*)-(+)-spiroketal and 2(*S*)-(−)-spiroketal dissolved in acetone; N,N-diethyl-3-methylbenzamide (DEET) (Sigma-Aldrich, Milwaukee, WI, USA) was also included as the standard. A 75 mg amount of each spiroketal or DEET was measured and placed in a separate 2-dram vial with 2 ml of acetone and muslin cloth, resulting in an acetonic solution. The removed cloth has a 0.750 mg/cm^2 concentration, and this was the highest concentration tested. Serial dilutions were carried out on each initial vial, and additional sections of muslin cloth were added to produce concentrations of 0.375, 0.187, 0.094, 0.047, 0.23, 0.011, and 0.005 mg/cm^2. Volunteers tested the treated cloth according to exact procedures of (Katritzky et al., 2010). The lowest concentration at which the repellent passed is an estimate of the Minimum Effective Dosage (MED).

Three male and one female volunteers participated in the repellency tests. During the test, all volunteers participated at the same time, each with their own cage. Since only two spiroketals were tested, patches of the same concentration of each enantiomer were tested by two of the volunteers, while the other volunteers were not testing. The patches then rotated to the next volunteer. In this manner, no patch was tested later than about 4 min from the first test. DEET was tested in a separate rotation from the spiroketals. All subjects provided informed written consent. The protocol was approved by the University of Florida Human Use Institutional Review Board-01 (Study #636-2005).

Olfactometer Bioassays A dual-port triple cage olfactometer was used to assess relative attraction and attraction-inhibition to treatments placed in separate test ports (Posey et al., 1998; Bernier et al., 2007b). The unit was constructed such that each cage is operated non-concurrently from the other two, and therefore, mosquitoes in the cage can either select to fly into one of the two ports or remaining in the cage. Approximately 75 (± 10) mosquitoes were placed in each cage 50 (± 10) min prior to experiments. Air was drawn in from the outside, filtered, and conditioned to 27±1 °C

and 60±5 % RH with a velocity of 28±1 cm/s through the ports.

Experiments were designed to test treatments in a competitive manner, where each spiroketal enantiomer added to the port with a synthetic human volatile blend compared to the synthetic human blend in the adjacent port. The stock blend consisted of 0.6 gL-(+)-lactic acid and 100 µl methyl disulfide dissolved in 250 ml acetone (Bernier et al., 2007a). A 500 µl aliquot of the stock solution was placed in a 1.4 ml plastic cap (15 mm i.d.×9.5 mm height) and 50 µl of either the 2(*R*)-(+) or 2(*S*)-(−)-spiroketal were added in a separate 400 µl cap (9 mm i.d.×9 mm height) to one of the ports; caps were placed on a 13×7 cm aluminum tray prior to insertion into the olfactometer port. Each test was 3 min in duration. The selection of cage and port test order was randomized, but the overall experiment designed such that each port within the 3 cages received a specific combined treatment only once. Therefore, 6 replicates of each competitive combination were obtained.

Results

The defensive spray of *A. glaber* is whitish in color, and can be sprayed carefully in the direction of the potential predator attack. Approximately 1 µl of an initial sample of this substance (*Asceles* 1) was extracted with benzene-d_6 for NMR analysis (unpublished results). Also, 1 µl of the same sample was extracted with approximately 100 µl of dichloromethane for GC/MS analysis. For a description and definitions of the *Asceles* sample numbers used, see Methods. From here on that nomenclature will be used to refer to specific samples of *A. glaber* defense spray. Preliminary GC/MS analysis showed one major compound that comprised over 95 % of the chromatogram for all samples (Supplemental Material Figs. S1, S2, S3, S4, S5, S6 and S7). The EI mass spectrum of the tentative minor natural spiroketal **2** (Supplemental Material Fig. S2 C), from the analysis of *Asceles* 2, matched well to a known spiroketal, 2-ethyl-,(2R-trans)-1,6-dioxaspiro[4.5]decane (Supplemental Material Fig. S2 5d; CAS# 76495-09-5), with a reverse-fit score of 891 and a probability score of 94.7 (Supplemental Material Fig. S6). In contrast, the mass spectrum of the major component (Supplemental Material Figs. S1, S2 and S3, Supplemental Material Fig. S4), did not have an equivalent in the National Institutes of Science and Technology (NIST) EI MS library database. The closest match for the major component was not a spiroketal, and it gave a reverse-fit score of below 700 (spiroketal **2** came next) (Supplemental Material Fig. S7). Based on visual inspection of the mass spectrum and its similarity to one found in the literature spiroketal **1** (Tengö et al., 1982), with additional structural information provided by NMR analysis, spiroketal **1** was synthesized and compared to the natural material by both GC/MS and NMR (Fig. 3; Supplemental Material Fig. S8). Identical mass spectra acquired for synthetic spiroketal **1** and the major component from *A. glaber* chemical defense spray provided confirmation of its identification (Supplemental Material Figs. S1 and S2 a and b).

For confirmation purposes and biological assays, both enantiomers of the proposed spiroketal were produced synthetically (Whitaker, 2012). The synthetic spiroketals exhibited roughly equal and opposite optical rotations, and thus are referred to as either a "2(R)-(+)-spiroketal" or "2(*S*)-(−)-spiroketal" as a correlation to the sign of the optical rotation. The enantiomers also displayed unique retention times when subjected to GC analysis utilizing a chiral stationary phase. Spectroscopically, the synthetic spiroketals were identical to data published in previous synthetic efforts (Ghosh et al., 2006). The details of the syntheses will be published separately.

For final verification of the identification for spiroketal **1**, approximately 5 µl of *Asceles* 5 was extracted with 100 µl of benzene-d_6 and a separate 5 µl was dissolved in 100 µl of D_2O for exhaustive NMR analysis to identify the major components (Fig. 3 and Supplemental Material Figs. S8 and S9). One-dimensional (1D) NMR spectral stack plots (Supplemental Material Fig. S8) as well as 2D TOCSY and HSQC spectral overlays (Fig. 3) of natural *A. glaber* defense spray (Supplemental Material Figs. S8 and S9) and synthetic spiroketal **1** (Supplemental Material Figs. S8 and S10) provide robust verification that the major component in that species' chemical defenses is spiroketal **1**. Additionally, the chemical shifts of ^{1}H and ^{13}C resonances observed in all spectra of synthetic spiroketal **1** in benzene-d_6 match closely with those published by Ghosh et al. measured for the same molecule dissolved in chloroform-d (Ghosh et al., 2006).

To determine the absolute configuration of spiroketal **1** as it occurs naturally in *A. glaber*, GC/MS analysis utilizing an enantiomer selective column was performed on the natural material in sample *Asceles* 2 and both synthetic enantiomers of spiroketal **1**. GC/MS results are shown in Fig. 4. The standards were found to be a mixture of two spiroketals and are labeled based on the predominant isomer (Fig. 4). Retention times confirmed that the major sporiketal in *A. glaber* defense spray is the 2(*S*)-(−) enantiomer (Fig. 4). Both peaks at 12.28 min and all three peaks at 12.39 min have identical EI mass spectra (Supplemental Material Fig. S11).

In preparation for ant and mosquito bioassays, the concentration of spiroketal **1** in natural *A. glaber* defense spray was determined by GC/MS utilizing serial dilutions of both natural and synthetic standard material. Integration of peaks from the TICs and molecular ion of all *Asceles* samples (Supplemental Material Fig. S1) along with those of a series

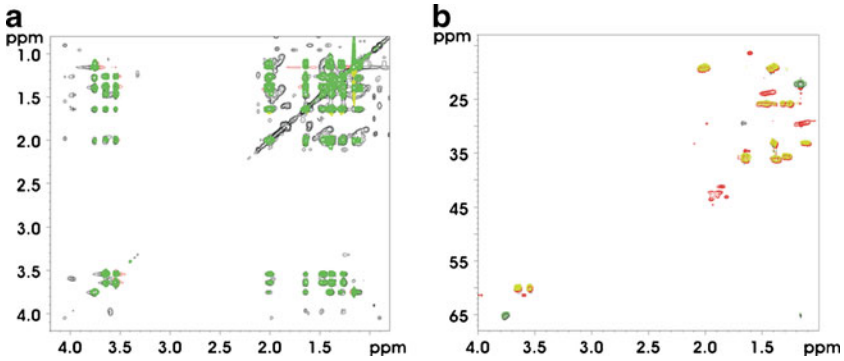

Fig. 3 Two dimensional (2D) NMR spectral overlays of: **a** synthetic spiroketal (1) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) dissolved in benzene-d_6 (*green and yellow*)on top of ^{1}H-^{1}H TOCSY spectra of *Asceles glaber* defense spray extracted with benzene-d_6 (black and red); **b** synthetic spiroketal (**1**) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) dissolved in benzene-d_6 (*green and yellow*)on top of ^{1}H-^{13}C HSQC spectra of *Asceles glaber* defense spray extracted with benzene-d_6 (*black and red*)

of dilutions of synthetic spiroketal **1** were compared via standard plot (Supplemental Material Figs. S12 and S13) to calculate the concentrations of that compound in *A. glaber* chemical defense spray reported in Table 1 (see also Supplemental Material Figs. S12 and S13) for the calibration curve and the extended table.

Having verified the identity of spiroketal **1** by GC/MS, NMR and by comparison with authentic synthetic standards, as well as having determined its concentration in *A. glaber* defense spray via GC/MS, its identity in the aqueous environment can be deduced by overlaying spectra (1D ^{1}H as well as 2D ^{1}H TOCSY) of natural and synthetic material both dissolved in D_2O, as shown (Fig. 5, Supplemental Material Fig. S14). All other NMR spectra for both of those samples also showed that all resonances and correlations present in spectra for the sample of synthetic spiroketal **1** matched an identical set of the same in spectra of the natural *Asceles* defense spray sample (*Asceles* 5), all collected in D_2O (Fig. 5, Supplemental Material Figs. S14, S15, S16 and S17). In addition to spiroketal **1**, spectra for the natural sample dissolved in D_2O revealed the presence of sugar-like resonances and correlations (Fig. 5, Supplemental Material Figs. S14 and S15). Since glucose has been reported from several phasmid chemical defense sprays to date (see Discussion), it was chosen as the most likely candidate for the identity of the *A. glaber* defense spray sugar. Indeed, as show in Fig. 5, overlaying spectra of pure authentic commercially obtained glucose show an exact match for glucose (also see Supplemental Material Figs. S14, S15, S16 and S17; S17 contains NMR spectra of an authentic sample of D-Glucose). Thus, *A. glaber* defense spray contains both spiroketal **1** and glucose.

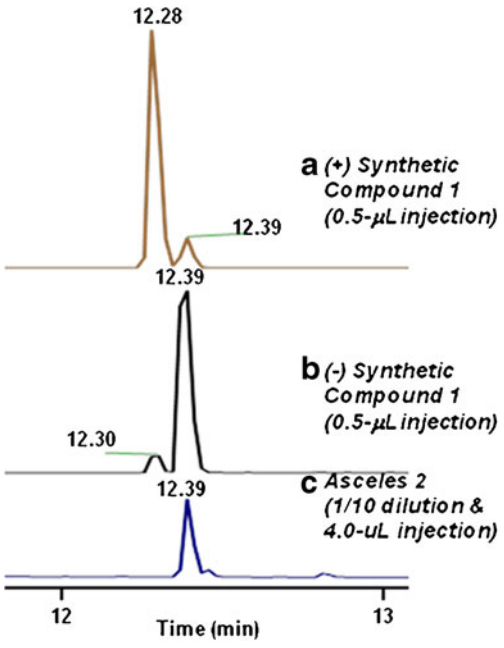

Fig. 4 The overlapping TICs (from the Beta DEX 120 column) for the 2(*R*)-(+) and 2(*S*)-(−) optical isomers of synthetic compound **1** (**a** & **b**) and for *Asceles* **2** (**c**) are normalized against the peak height of the 2(*R*)-(+) optical isomer

We also determined the efficacy of these compounds against potential arthropod predators and other arthropods. In one set of experiments, the compounds were tested for their ability to repel red imported fire ants (*Solenopsis invicta*) as a model predator. Two bioassays were used to evaluate the repellent activity of the spiroketal against *S. invicta*. The Y-tube olfactometer measures the response of worker ants to the spiroketal in the air stream of one choice arm vs. the solvent control in the other choice arm of the olfactometer. The

Table 1 GC/MS quantification: concentration of compound 1 (µg/µl) in various *Asceles* samples (*N*=3)

Samples	Average	Std. Deviation
Asceles-1	11	0.15
Asceles-2	42	1.4
Asceles-3	3.5	0.09
Asceles-4	4.5	0.11

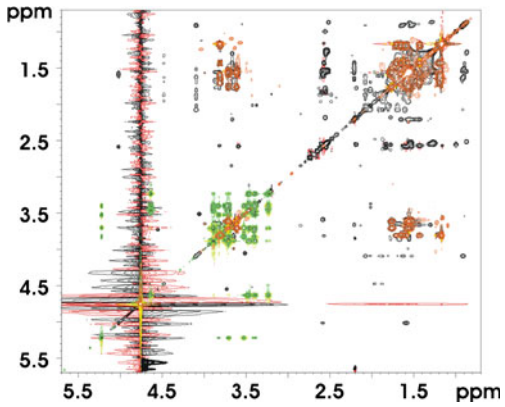

Fig. 5 Identification of Glucose in *Asceles glaber* defense spray: Two dimensional (2D) ^{1}H-^{1}H TOCSY spectra of *Asceles glaber* defense spray (bottom spectrum: *black and red*), synthetic spiroketal (**1**) ((2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane) (top spectrum: *orange and light yellow*) and authentic commercial glucose (*green and greenish-yellow*). All spectra of samples dissolved in D_2O

positive control, queen recognition pheromone, was always highly attractive (mean ± SE=77.5±1.4 %; X^2=12.1, 1*df*, two-tailed *P*=0.001, *N*=3). The results are shown in Fig. 6a as the percent worker ants responding to the spiroketal. Significant repellent activity was only found in the 3.3 % spiroketal

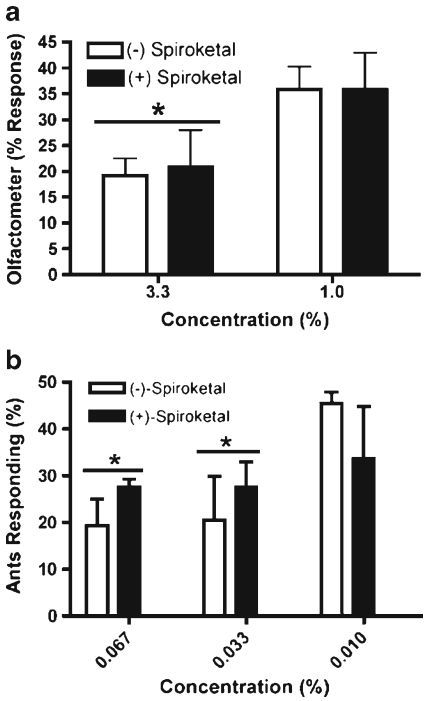

Fig. 6 **a** The Y-tube olfactometer response of fire ant workers to the spiroketal at the percent concentration indicated (pentane, W/V, mean ± SE; *n*=3). A mean response <35 % indicates significant repellent activity (*). Only the 3.3 % concentration showed repellent activity, **b** Contact repellency bioassay results (mean ± SE; *N*=3) for a series of Spiroketal dilutions (% concentration, W/V, in pentane). All concentrations were significantly different (repellent) from the pentane control, except the 0.01 % concentration. (see Results)

concentration (Fig. 6a, mean ± SE=29.2±3.6 %, *N*=3; X^2=6.4, 1*df*, two-tailed *P*=0.011). In previous work that investigated possible repellents against fire ants, the initial concentration of test compounds was 1.0 %. If the compound did not show repellent activity at 1 %, it was dropped from further consideration (Vander Meer et al., 1996). Based on the repellent activity of the spiroketal in the olfactometer bioassay, the compound has activity only at >1 %, or poor repellency to fire ant workers. Since some compounds can exhibit contact repellency (as well as or in addition to through the air repellency), we subjected the spiroketal to a bioassay that measured its effectiveness at preventing worker ants from feeding on a food substance. Compounds that are good volatile repellents can confound this bioassay, since the ants would be prevented from contacting the test compound. Our results (Fig. 6b) demonstrate that the spiroketal is an excellent contact repellent for fire ants. Activity is lost only between 0.033 and 0.01 %, which is 100 times more effective than demonstrated by the olfactometer bioassay, thus through the air activity is unlikely. In addition, observation showed that the ants approached and touched the test material before moving away. All evaluated concentrations (1.0 % though 0.01 % were significantly repellent to fire ant workers (above 0.067 % not shown), except the 0.01 % concentration (Fig. 6b). Therefore, by inference 1 % through 0.033 % activities also were significantly repellent. The dichotomy in results for the two bioassays points out the importance of differentiating contact and through the air repellency.

In addition to the testing of the synthetic spiroketals on ants,, their effects on mosquitoes (*A. aegypti*) also were studied. The repellency of the 2(*R*)-(+) and 2(*S*)-(−)-spiroketal were nearly identical at about 0.500 mg/cm^2. The amount of spiroketal required to repel *A. aegypti* was about 100 times higher than the amount of DEET required (Table 2). The 2(*R*)-(+)-spiroketal suppressed attraction to a synthetic human volatile blend when combined with the blend and tested again in a port containing only the blend. In contrast, the (−)-spiroketal increased the attraction of mosquitoes to the side containing it and the blend (Table 3).

Discussion

The data presented in this report demonstrate that the defensive spray of *Asceles glaber* contains primarily (2S,6R)-(−)(*E*)-2-methyl-1,7-dioxaspiro(5,5)-undecane (spiroketal **1**), 2-ethyl-1,6-dioxaspiro[4.5]decane (spiroketal **2**), and glucose. Additionally, the data show that spiroketal **1** is able to repel red imported fire ants (*Solenopsis invicta*) at concentrations well within the range of those deployed in the chemical defense system of *A. glaber,* and it exhibits a

Table 2 Minimum effective doses (MED)[a] of 2(*R*)-(+) and 2(*S*)-(−)-spiroketal and DEET tested on human volunteers against *Asceles aegypti* mosquitoes

Volunteer No.	2(*R*)-(+)-spiroketal	2(*S*)-(−)-spiroketal	DEET
1[b]	0.094	0.375	0.005
2	0.750	0.750	0.003
3	0.375	0.187	0.005
4	0.750	0.750	0.005
Average (±SE)	0.492 (0.159)	0.516 (0.141)	0.005 (0.001)

[a] MED in mg/cm^2 ; ≥ 5 bites in 1 min exposure to 500 mosquitoes is considered a failure at that compound concentration

[b] Volunteer 1 was a female, the other three volunteers were males

behavioral response from mosquitoes (*Aedes aegypti*). Chemical defense sprays from the same glands in other phasmid species have been shown by others to be effective repellents against potential predators such as ants, beetles, mice, rats, frogs, and birds and thus are assumed to be used for defense (Eisner, 1965; Carlberg, 1985a, 1985b, 1986; Dossey, 2011). However, it is unknown how effective spiroketal **1** is at protecting *A. glaber* in the wild.

Given the biological assay results described above and in previous literature, several conclusions can be made about the mechanism by which the chemical defenses of stick insects, particularly *A. glabor*, function. First, this spray appears to function as a contact repellent, not a volatile odor repellent. For both fire ants and mosquitoes, it is clear that the major spiroketal from *A. glabor* defense spray is a very weak airborne repellent. However, fire ants are turned away quite effectively upon contact with the substance, and it also appears to deter mosquitoes from landing and/or feeding. Additionally, the literature (and Dossey, personal observation) demonstrate that other stick insect defense sprays, like those of *Anisomorpha buprestoides* (mentioned previously), are quite irritating primarily when they come into contact with the eyes, nose, and mouth where sensitive mucus membranes are preset, They also produce a generally irritating response in model predators tested as well as humans and dogs (Dziezyc, 1992; Eisner et al., 1997; Paysse et al., 2001; Dossey, 2010; Brutlag et al., 2011). Thus, stick insect defense sprays such as this appear to function primarily as contact repellents, which are generally irritating to predators.

Table 3 Attraction of female *Asceles aegypti* mosquitoes to a synthetic blend compared to the blend with each spiroketal in a dual-port olfactometer

Only Blend	2(*R*)-(+)-spiroketal and Blend	Only Blend	2(*S*)-(−)-spiroketal and Blend
20.4(3.6)	8.7(3.9)	16.4(6.7)	33.1(3.7)

Attraction is presented as a percentage ± (SE) of approximately 75 female *A. aegypti* that were trapped in each port containing the treatments. Mosquitoes may select to fly into either treated port or to remain in the cage (6 replications were conducted for each spiroketal and blend vs. blend combination)

Since it is unlikely that mosquitoes prey upon *A. glaber*, it would not be expected that the defense secretions and the components contained therein would be mosquito-specific repellents. The weak repellency shown by the spiroketals indicate a deterrence to mosquito landing and biting behavior that could perhaps be a result of these compounds being irritating to the mosquito. While the repellency Minimum Effective Dosage (MED) values of the 2(*R*)-(+) and 2(*S*)-(−)-entantiomers are approximately 0.500 mg/cm^2, the amount of DEET required to repel these mosquitoes is 0.005 mg/cm^2 (100 times lower) (Table 2). However, DEET is known to be potent at low concentrations, typically ranging from 0.011 to 0.094 mg/cm^2 in this type of assay. Thus, the spiroketals identified from *A. glaber* may function as a general irritant to many other arthropods as well as other animal predators. Additionally, Dipterans have been observed as both endo- and ectoparasites on various species of stick insects (Neff and Eisner, 1960; Tilgner and McHugh, 1999). Therefore, these compounds may also affect Dipterans as well as other arthropods posing a threat to stick insects. These hypotheses require further investigation.

While the contact repellent efficacy appeared to be identical for both enantiomers of the spiroketals, there was a noticeable difference between the 2(*R*)-(+) and 2(*S*)-(−) forms when examined for their volatile based effects in competitive assays using a dual-port triple cage olfactometer (Table 3). The 2(R)-(+)-spiroketal decreased the attraction of mosquitoes to the ports that contained this enantiomer, whereas the 2(*S*)-(−)-spiroketal increased the port catches. This result is interesting since no clear difference was observed in contact repellency between the two. However, it has been established previously that there can be significant differences in the behaviors determined as evident in results when using these different types of bioassays (Weldon et al., 2011). Thus, while both enantiomers may show an irritant effect to mosquitoes at a higher concentration then DEET, there apparently is still some mechanism by which host-seeking mosquitoes are differentiating between the two spiroketal forms at a distance of a few meters down to several centimeters.

Interestingly, the spiroketals analyzed in this study are utilized as pheromones by a number of other insect species (Francke et al., 1977; Tengö et al., 1982; Francke and Kitching, 2001; Ghosh et al., 2006; Booth et al., 2009). Additionally, the amounts secreted by *A. glaber* in response to being disturbed are quite small compared to other species with more robust chemical defenses such as in the genera

Anisomorpha, *Peruphasma*, *Megacrania* and others (Eisner, 1965; Smith et al., 1979; Chow and Lin, 1986; Ho and Chow, 1993; Dossey et al., 2006, 2008; Dossey, 2010, 2011). In fact, it has been postulated that phasmids may also utilize their chemical defense systems for other purposes such as pheromonal communication (Tilgner, 2002; Dossey et al., 2008, 2009). In general, several other arthropods are known to utilize their various chemical production systems for multiple purposes, a concept described by Blum as Semiochemical Parsimony (Blum, 1996). Thus, it is possible that *A. glaber*, as well as other phasmids, use these compounds as pheromones or in other forms of chemical communication.

Including the current report, glucose has been found in the defense spray of 6 species of phasmids to date including: *Anisomorpha buprestoides* (Dossey et al., 2006), *Peruphasma schultei* (Dossey et al., 2006), *Parectatosoma mocquerysi* (Dossey et al., 2007), *Phyllium westwoodii* (Dossey et al., 2009), *Megacrania nigrosulfurea* (Prescott et al., 2009), and *Asceles glaber*. It also has been shown that the phasmid *A. buprestoides* can biosynthesize its defensive spray monoterpene *de novo* from ^{13}C-labeled glucose (Dossey et al., 2008). Additionally, beetles (Order Coleoptera) of the family Chrysomelidae (the leaf beetles) use precursors that are conjugated with glucose for larval defensive secretion biosynthesis and transport (Kunert et al., 2008). The chemical simplicity of phasmid chemical defense sprays and lack of other common primary metabolites (amino acids, etc.) besides glucose suggests that glucose plays a critical role in their chemical defense system. By analogy to the pathways elucidated in Chrysomelid beetles so far, glucose likely plays a similar role in phasmids as it does in beetles. The presence of glucose in *A. glaber* defense spray, as well as in that of other phasmid species, suggests that phasmids may utilize glucoconjugate precursors of multiple classes of secondary metabolites for biosynthesis, transport, or both. These hypotheses require further investigation.

The prothoracic exocrine glands of the Phasmatodea represent one key synapomorphy that defines this insect group (Tilgner, 2002; Bradler, 2009). Chemical analyses have shown that the composition of their secretory product differs significantly among the phasmid lineages studied to date (see Introduction), suggesting a potential chemotaxonomic value of the semiochemicals expressed. *Ascales glaber* is a typical representative of the Subfamily Necrosciinae, which also includes *S. sipylus*. This latter species is, according to Bouchard et al. (1997), apparently characterized by a complex mixture of chemicals, comprising limonene, benzothiazole, benzaldehyde, acetic acid, and predominantly diethyl ether as the major compound. The spiroketal here reported from *A. glaber* also has two ether linkages. However, this is not a strong similarity, and we believe that diethyl ether may have been an artifact in the report about *S. sipylus* secretion, since it is both unusual to find it in an animal and it is also a common laboratory solvent. At least, the spray of that species merits re-investigation, using other analytical methods. At any rate, the composition of defensive secretions is notably different in the two Necrosciinae species analyzed so far. This underlines the heterogeneous nature of this phasmid assemblage, which is also evidenced by the analysis of some morphological characters (Sellick, 1997; Bradler, 2009).

In summary, we have demonstrated the stereo-specific identification of spiroketal **1**, as well as glucose, in the chemical defense spray of *Asceles glaber* as well as its effect on ants and mosquitoes. We also have provided a well-supported mechanism by which this and possibly other stick insect chemical defenses function to ward off potential predators, attackers, and sources of other offending stimuli. These results demonstrate that spiroketals such as these found in the defense spray of *A. glaber* merit further investigation as potential components of insect repellents. In general, this report lends support to the demonstration of the chemical biodiversity that exists in insect chemical defense systems as well as the utility of stick insects as models of studying those systems.

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Supplemental Material Available online: S1) GC/MS Chromatograms of all natural samples of *Asceles glaber* defense spray analyzed; S2) Mass spectra of *A. glaber* defense spray samples and synthetic spiroketal **1**; S3) TIC for *A. glaber* chemical defense spray; S4) The EI mass spectra for *A. glaber* chemical defense spray; S5) TIC and mass spectrum of synthetic spiroketal **1**; S6) The NIST EI Mass Spectral Library search identified the minor peak in *A. glaber* defense spray; S7) The NIST EI Mass Spectral Library search for the major peak for *A. glaber* chemical defense spray; S8) 1D ^{1}H NMR spectral overlays of natural *A. glaber* defense spray and synthetic spiroketal **1** In benzene-d_6; S9) NMR spectra of *A. glaber* defense spray extracted with benzene-d_6; S10) NMR spectra of synthetic spiroketal **1**; S11) EI mass spectra from enantiomer selective GC/MS analysis of *A. glaber* chemical defense spray and synthetic spiroketal **1**; S12) External calibration curves from the GC/MS of synthetic spiroketal **1** for quantification of that compound in *A. glaber* chemical defense spray; S13) Extended table of concentrations of spiroketal **1** in *A. glaber* chemical defense spray; S14) 1D ^{1}H NMR spectral stack plots of natural *A. glaber* chemical defense spray and authentic D-Glucose dissolved in D_2O, S15) NMR spectra of *A. glaber* defense spray dissolved in D_2O; S16) NMR spectra of synthetic spiroketal **1** dissolved in D_2O; and S17) NMR spectra of authentic D-Glucose dissolved in D_2O.

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Regeneration of Complex Oil-Gland Secretions and Its Importance for Chemical Defense in an Oribatid Mite

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Abstract Most oribatid mites possess a pair of opisthonotal exocrine glands that produce mostly complex, species-specific secretions. Such blends may contain more than 10 different compounds, but hardly anything is known about their primary biosynthesis or regeneration. I analyzed recovery of the 6 main components from the 11-compound secretion of the oribatid mite *Archegozetes longisetosus* Aoki, including the main chemical classes hydrocarbons, aromatics, and terpenes, during a 20-day time course after complete gland depletion. About 10 % of the original total secretion amount was restored after 24 hr, and after 2–6 days, the amount had reached the range of total amount observed in the control group. Most compounds were recovered at similar rates within the first 48 hr. An important exception was pentadecane, which was predominantly produced in the first few hours, suggesting that this compound is the main solvent of the secretion. Although relative amounts of the main compounds differed significantly over time, the complex profile of the whole secretion was stable and not confidently distinguishable among the sampling dates. The general recovery rate was high during the first 48 hr, about 25 times higher than in the remaining 18 days. The biological importance of this high initial investment was supported by predation experiments: the predacious rove beetle *Stenus juno* was first repelled after 48 hr when at least 25 % of secretions was restored.

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Introduction

Many insects and chelicerates discharge defensive secretions in response to predator attack or disturbance (reviewed in e.g., Eisner, 2003), but little is known about the production aspects of the underlying glandular systems, such as primary biosynthesis (Happ and Meinwald, 1965; Farine et al., 2000; Schulz, 2001; Morita et al., 2004; Noge et al., 2005) or the dynamics of regeneration of secretions (Fescemyer and Mumma, 1983; Baldwin et al., 1990; Farine et al., 2000; Tomita et al., 2003; Raspotnig, 2006). However, these parameters are important for an understanding of costs of secretory production and benefits of their defensive use (Baldwin et al., 1990).

Most sarcoptiform mites (glandulate Oribatida, including Astigmata) possess a large pair of exocrine opisthonotal glands, also termed oil-glands (Raspotnig, 2010). The chemical composition and biological function of secretions from these glands have been well-studied in Astigmata over almost 4 decades (reviewed in Kuwahara, 2004), and knowledge of oil-gland chemistry in other oribatid mites has accumulated in the last 15 years (see Raspotnig et al., 2011a for a recent summary). Oil-gland secretion profiles are mostly species-specific mixtures of hydrocarbons, aromatics, terpenes, and sometimes alkaloids, and it has been suggested that these profiles might be a valuable set of phylogenetically and taxonomically informative characters (e.g., Sakata and Norton, 2001; Heethoff et al., 2011a; Raspotnig et al., 2011b). Our relatively broad knowledge of oil-gland chemistry in sarcoptiform mites is contrasted by lack of information regarding the dynamics of secretion biosynthesis. Only two studies of post-deplection regeneration exist, and they seem inconsistent.

Tomita et al. (2003) reported that all compounds in the oil-gland secretions of the astigmatid mite *Tyroborus lini* Oudemans were regenerated at similar rates and within 60 hr, except for neryl formate, which was synthesized with a delay of several days. By contrast, Raspotnig (2006) found that secretions in the oribatid mite *Collohmannia gigantea* Sellnick were not regenerated to any detectable degree, even after 14 days.

To clarify the apparent contradiction, I investigated the regeneration of oil-gland secretions after artificial depletion in the model oribatid mite *Archegozetes longisetosus* Aoki. This species was used because its complex oil-gland secretions contain a specific set of terpenes, aromatics, and hydrocarbons, the so-called 'Astigmata-compounds' (sensu Sakata and Norton, 2001; Heethoff et al., 2011a), which are considered synapomorphic for the large clade of glandulate Oribatida that includes the Astigmata (Norton, 1998). In detail, the oil-gland secretion of *A. longisetosus* consists of 2-hydroxy-6-methyl-benzaldehyde (2,6-HMBD), 3-hydroxybenzene-1,2-dicarbaldehyde (γ-acaridial), (*E*)-3,7-dimethyl-2,6-octadienal (geranial), (*Z*)-3,7-dimethyl-2,6-octadienal (neral), (*Z*)-3,7-dimethyl-2,6-octadienyl formate (neryl formate), tridecane, pentadecane, pentadecene, heptadecane, heptadecene, and heptadecadiene (Heethoff and Raspotnig, 2011). Hence, secretions of *A. longisetosus* share 6 compounds with *C. gigantea* (Raspotnig, 2006) and also 6 of the 11 compounds of *T. lini* (Tomita et al., 2003), and thus represents a well-suited model system for understanding general properties of oil-gland secretion biosynthesis in sarcoptiform mites.

Here, I show that: (i) in contrast to *C. gigantea*, *A. longisetosus* can restore their full set of oil-gland secretions within some hours/days; (ii) pentadecane seems to be the main solvent of the secretion of *A. longisetosus*; (iii) some main compounds were recovered at different rates; but (iv) the complex composition of the secretion was quite stable throughout the whole time of recovery; and (v) about one-quarter of the secretion volume usually contained in the oil-gland reservoirs is needed for effective chemical defense against a large predator (the rove beetle *Stenus juno* Paykull).

Methods and Materials

Animals The laboratory strain *A. longisetosus* ran (Heethoff et al., 2007) was cultured in constant dark at 28 °C, as described in Heethoff and Raspotnig (2011). Adult specimens were sampled if they had fed and were actively moving. The rove beetle *Stenus juno* was chosen as a model predator for chemical defense because it feeds on *A. longisetosus* when their oil-glands are empty, but is repelled when oil-glands are filled (Heethoff et al., 2011b; Heethoff and Raspotnig, 2012a).

Experimental Depletion of Oil-Glands Discharge of oil-glands was evoked by dipping 10 groups each with 50 adult specimens into hexane for 60s, followed by 1 hr of recovery, and then a second cycle of 60s in hexane (following the HRH-protocol of Heethoff and Raspotnig, 2012b). This treatment leads to reproducible depletion of oil-glands without influencing life-history parameters (Heethoff and Raspotnig, 2012b) or affecting morphology and ultrastructure of the glandular tissues (Paavo Bergmann, personal communication, Fig. 1).

Maintenance and Sampling after Oil-Gland Depletion Specimens were maintained in 10 different groups under conditions similar to those of the source culture: in constant dark at 28 °C and saturated air humidity in plastic boxes (10×8×5cm) with a floor covering of 1 cm gypsum/charcoal mix (6:1). The unicellular alga *Chlorella* (as commercially available powder) was provided as food, as in the culture, and offered/removed on filter paper (3×3cm) every 2 days. At 5, 10, 24, and 48 hr and 6 and 20 days after the depletion, mites were sampled for chemical analysis and predation bioassay (see below); a fresh piece of *Chlorella*-covered filter paper was provided in each of the 10 boxes and collected when 2–4 specimens had actively invaded the new resource (~10 min). In this way, mechanical disturbance of specimens during the collection procedure was minimized. From the 30–40 collected specimens pooled from the 10 boxes at each sampling time, 20 were randomly chosen for analyses and remaining specimens were discarded.

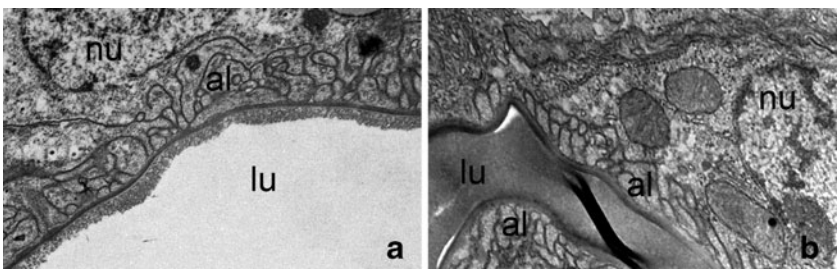

Fig. 1 Ultrastructure of the oil-gland-tissue of *Archegozetes longisetosus*, as observed by transmission electron microscopy. (**a**) control specimen, (**b**) HRH-treated specimen with completely depleted oil-gland reservoirs. Glandular cells most probably represent class I cells (Noirot and Quennedy, 1974). Except for the collapse of the reservoir lumen no differences were apparent. al: apical labyrinth, lu: lumen of the oil-gland reservoir, nu: nucleus

Extraction of Oil-Gland Secretions At each sampling time, 10 of the 20 sampled mites were individually transferred with a fine brush into 50 μl of hexane, to release oil-gland secretions into the solvent (e.g., Heethoff and Raspotnig, 2011). After 3 min, specimens were removed, and 130 ng of 6-methyl-5-hepten-2-one (MHO, Sigma-Aldrich, Vienna) were added to the raw extract as an internal standard for quantification purposes. As a control, 10 untreated specimens from the source culture were individually extracted and analyzed.

GC/MS and Quantification A trace gas chromatograph (GC) coupled to a DSQ I mass spectrometer (MS; both from Thermo, Vienna, Austria) and equipped with a ZB-5MS fused silica capillary column (30 m long, 0.25 mm diam, 0.25 μm film thickness, Phenomenex, Germany) was used for the analyses. Injection was splitless with helium (at a constant flow rate of 1.2 ml/min) as a carrier gas. The column temperature was programmed from 50 °C (held for 1 min) to 200 °C at 10 °C/min, and then to 300 °C at 15 °C/min. The ion source of the mass spectrometer and the transfer line were kept at 200 °C and 310 °C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Absolute amounts of secretions were calculated based on the integration of peak areas in the chromatograms relative to a constant amount of the internal standard (5 ng MHO, see above), and thus expressed in % peak area of this standard. Relative amounts of compounds were calculated as % peak area of the whole secretion. Statistical analyses were based on the relative amounts of compounds accounting for more than 5 % of the secretion (referred to as "main compounds"): 2,6-HMBD, neral, neryl formate, γ-acaridial, pentadecane, and heptadecene. The remaining compounds (geranial, tridecane, pentadecene, heptadecane, and heptadecadiene) together added up to only about 5 % of the secretion amounts and it was not feasible to quantify these small amounts early in regeneration; they were excluded from statistical analyses, but seemed to recover with similar patterns.

Identification of Compounds The chemical composition of oil gland secretions of adult and untreated *A. longisetosus* has been described earlier (Heethoff and Raspotnig, 2011). For the present study, regenerated compounds were confirmed by comparisons of retention times and mass-spectra to the secretions and authentic standards used there.

Statistical Analyses Differences among restored secretion amounts and patterns of single compounds during regeneration were analyzed by ANOVA (log-transformed data were used where necessary); homogeneous groups were identified by *post-hoc* statistics (Tukey HSD) in SPSS 20. The existence of distinct chemical groups during the time course of secretion regeneration was evaluated by multivariate cluster analyses with UPGMA and single linkage (both using Euclidean distances) and with Ward's method in PAST 2 (Hammer et al., 2001).

Predation Experiments At 5, 10, 24, and 48 hr and 6 and 20 days after the depletion, 10 of the 20 sampled mites were collectively presented to 5 specimens of *S. juno* in a plastic container (10×8×5 cm) with a floor covering of 1 cm gypsum/charcoal mix (6:1) and observed for 30 min by eye under ambient light conditions to qualitatively test whether the quantity of newly synthesized secretions was high enough to attain chemical protection from predation (see Heethoff et al., 2011b for details of *Stenus*/*Archegozetes* predation tests). The beetles were always starved for 5 days before the experiments.

Results

Regeneration Secretion was already detectable after 5 hr, and amounts increased significantly with time ($F_{5,54}$=6.9, P< 0.001; Fig. 2). After 48 hr to 6 days, some specimens had regenerated secretions amounts within the range of the control group, but even after 20 day, total amounts of some specimens were still significantly lower ($F_{1,18}$=33, P<0.001; Fig. 2). The control group of untreated mites contained secretion amounts of 452 % (=4.52 times the amount of the internal standard MHO, range 201–664 %, SD: 131); after 5 hr, the amounts averaged 14 % (3–33 %, SD: 12); after 10 hr, the average was 31 % (8–55 %, SD: 15); and 46 % (9–98 %, SD: 30), 105 % (4–199 %, SD: 71), 121 % (26–260 %, SD: 79), and 141 % (2–315 %, SD:108) after 24 hr, 48 hr, 6 days, and 20 days, respectively (Fig. 2). While all main compounds showed significantly different relative amounts during regeneration, the patterns were only consistent for neral (steadily increasing relative amounts) and pentadecane (high relative amounts at the beginning, lower and constant values for the rest of the time course; Table 1). There was no apparent delay in the initial recovery for any compound (Fig. 3). Pentadecane, representing 6 % of the control secretion, was recovered with relative amounts of 40 % after 5 hr, 14 % after 10 hr, and then 8–9 % for the rest of the experiment (Fig. 4), which is a reduction by a factor of 4, while its total amounts increased by a factor of 3 from 4 % (5 hr) to 12 % (20 day) during the time course (Fig. 3). Recovery rates of some of the main compounds differed during the first 48 hr: 2,6-HMBD, neral, neryl formate, γ-acaridial, heptadecene, and pentadecane were restored with rates of 0.29, 0.29, 0.67, 0.33, 0.09, and 0.22 % of MHO per hour, respectively. Hence, neryl formate was produced with higher, and heptadecene with lower rates than the other main compounds. In the remaining time (432 hr), 2,6-HMBD, neral, neryl formate, γ-acaridial,

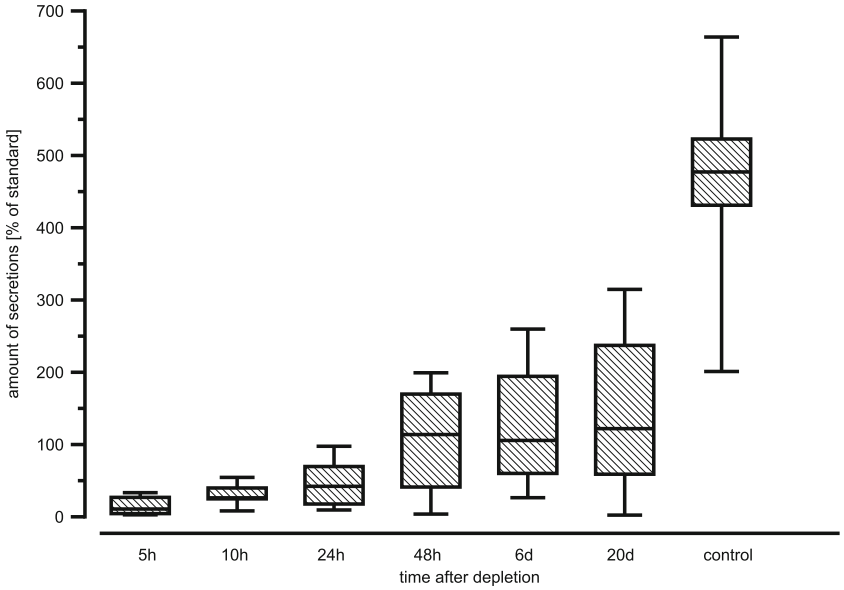

Fig. 2 Recovery of total amount of oil-gland secretion of *Archegozetes longisetosus* during the observed period of 20day. Amount of secretion increased with time ($F_{5,54}$=6.9, P<0.001), but did not reach the original level even after 20day ($F_{1,18}$=33, P<0.001)

heptadecene, and pentadecane were restored with rates of 0.01, 0.04, -0.02, 0.03, 0.01, and 0.003 % of MHO per hour, respectively. The biosynthetic rate per hour was on average ~25 times higher in the first 2 days (0.315 % of MHO/h) when compared to the remaining final 18 days (0.012 % of MHO/h).

Neryl formate represented the main compound during the first 6 days (except for 5 hr, when it was pentadecane), but after an initial increase, its absolute amount decreased from 48 hr to 20 days (Fig. 3). This decrease was paralleled by a conspicuous increase of neral. The connection of these two compounds (i.e., the same biochemical pathway/precursor) is supported by a strong linear negative correlation (R= -0.94, P<0.001; Fig. 5). Hence, I summed both amounts and treated them as an additional extra single component (Table 1, Figs. 3, 4). When analyzed together, the relative amounts of neral and neryl formate were constant during the recovery process, further indicating that they share a common biosynthetic pathway or precursor, or that neryl formate and neral might interchange (Table 1, Fig. 4).

Significant differences of the relative amounts of the main compounds among sample dates do not necessarily imply that the complex secretion as a whole differs significantly during the regeneration process. When relative amounts of main compounds are compared to the control secretion during regeneration, no apparent strong aberration appeared (compounds ranged between 0.4 and 2), except for pentadecane, which was 7 times higher in the first 10 hr (Fig. 6). Hence, I tested for the existence of distinct groups with a multivariate cluster analyses, including the relative amounts of main compounds in PAST 2 (Hammer et al., 2001). None of the sampling dates or the control were completely recovered as distinct clusters, neither using the paired-group, single linkage nor Ward's method, and irrespective of treating neral and neryl formate separately or as a single value (data not shown).

Predation Tests At 5 hr, 10 hr, and 24 hr after depletion, the feeding success rate of *S. juno* on *A. longisetosus* was more than 90 % (5 hr: 4 attacks, 4 fed; 10 hr: 6 attacks, 5 fed; 24 hr: 4 attacks, 4 fed). The single failed attack resulted from the labium not sticking properly to the prey; all attacks using mandibles were successful. After 48 hr, there were the

Table 1 Comparison of the relative amounts of main compounds during regeneration, indicating significant differences (ANOVA with F and P values) and homogeneous groups (Tukey HSD with P<0.05, indicated by same letters in rows)

component	$F_{6,63}$	P	5 hr	10 hr	24 hr	48 hr	6d	20d	con
2,6-HMBD	3.3	0.007	a	ab	b	b	ab	ab	ab
neral	6.6	<0.001	a	a	ab	abc	bc	c	ab
neryl formate (nf)	4.9	<0.001	ab	b	ab	b	ab	a	b
neral + nf	2.1	0.064	a	a	a	a	a	a	a
γ-acaridial	7.6	<0.001	ab	a	ab	ab	bc	ab	c
pentadecane	6.2	<0.001	a	b	b	b	b	b	b
heptadecene	2.5	0.032	a	b	ab	ab	ab	ab	ab

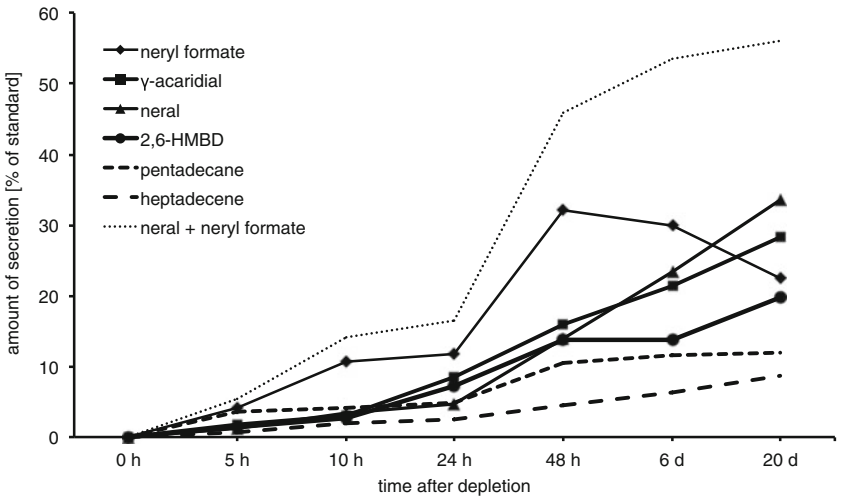

Fig. 3 Recovery of absolute amounts of the main compounds from the oil-gland secretion of *Archegozetes longisetosus*. Each point represents the average of 10 replicates

first indications of chemical defense: some mites caught by the beetles were immediately released, followed by typical cleaning behavior by the beetles (Heethoff et al., 2011b). However, feeding success was still quite high: of 10 attacks, 5 (50 %) were successful. The potential for chemical defense increased over time (40 % and 25 % of attacks successful after 6 days and 20 days).

Discussion

Regeneration In this study, I demonstrated for the first time that non-astigmatid oribatid mites can regenerate oil-gland secretions. Raspotnig (2006) detected no secretions in *C. gigantea* glands during a 14-day-period following depletion, but further experiments are needed to determine whether this is a general property of *C. gigantea* or a result of external factors, e.g., culturing methods and feeding after depletion. The latter is more likely since *C. gigantea* is not easily maintained in the laboratory, while good culturing conditions are well known for *Archegozetes* (e.g., Heethoff et al., 2007). The only studied astigmatid mite, *Tyroborus lini*, clearly can restore oil-gland secretions. Tomita et al. (2003) showed that neryl formate is restored with a delay of several days, and explained this as resulting from a higher cost of its synthesis. Since neral was recovered without delay, the supposed higher cost for neryl formate synthesis is questionable: both, neral and neryl formate are probably synthesized via isopentenyl-pyrophosphate via the mevalonate pathway (Morita et al., 2004; Noge et al., 2005) with geraniol as a general precursor (Noge et al., 2005) and geranyl pyrophosphate as the last common precursor in their biosynthesis (Schulz, 2001).

The strong negative correlation of neral and neryl formate observed in *A. longisetosus* suggests that one pathway is strongly preferred while the other is reduced. This does not, however, explain how the absolute amounts of neryl formate decreased while they increased for neral. One possible explanation for this observation would be that neral is not produced via geranyl pyrophosphate but via some pathway using neryl formate as educt. This would be in accordance with findings of Morita et al. (2004), that neryl

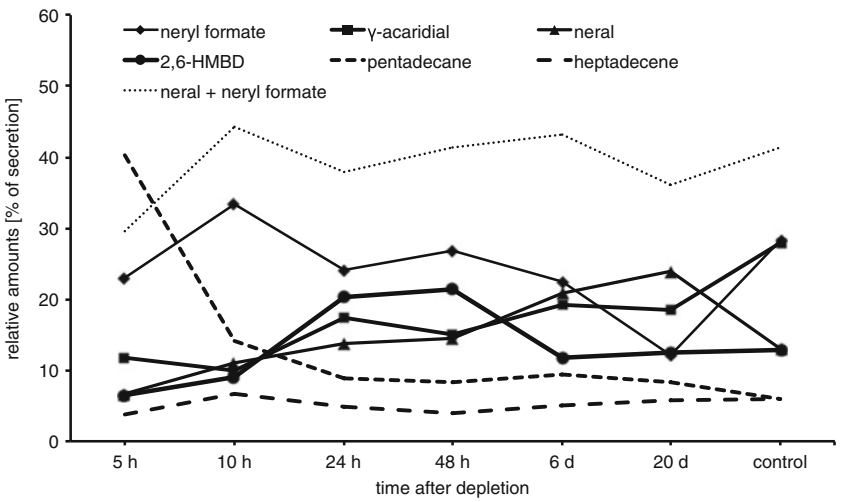

Fig. 4 Dynamics of the recovery of relative amounts of the main compounds from the oil-gland secretions of *Archegozetes longisetosus*. Each point represents the average of 10 replicates

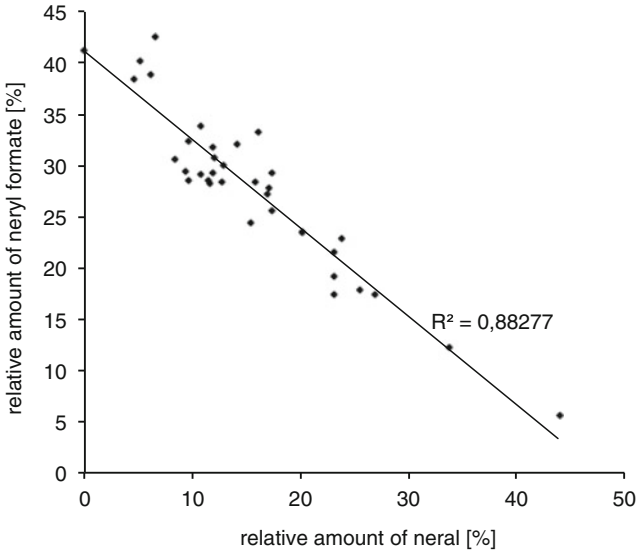

Fig. 5 Linear correlation of relative amounts of neral and neryl formate in oil-gland secretions of *Archegozetes longisetosus*

formate appears during the course of secretion synthesis in the astigmatid mite *Carpoglyphus lactis* Linnaeus, but is absent from the final secretions, which contained only neral. However, this hypothesis includes unlikely biochemical pathways and certainly needs further investigation. Alternatively, the synthesis of neryl formate might stop at a certain quantity, and the absolute amounts could be reduced by a permanent leakage of the glands, as has been demonstrated for the astigmatid mite *Rhizoglyphus robini* (Mizoguchi et al., 2003). This, however, does not explain the strong correlation of neryl formate and neral.

As Tomita et al. (2003) did not analyze the temporal recovery dynamics of the relative composition of oil-gland secretions in the astigmatid mite *T. lini* in detail, I can compare my results only to those for some insect species. Abdominal tracheal gland secretions in the roach *Diploptera punctata* Eschenholz are a mixture of three *p*-benzoquinones, and during regeneration these are produced not at similar rates but in a specific order over 45 days (Baldwin et al., 1990). Like *A. longisetosus*, some specimens of *D. punctata* did not refill the defensive glands after depletion. Regeneration in another roach, *Eurycotis floridana* Walker, was studied by Farine et al. (2000); after artificial depletion, males recovered the full amount of secretion in 30 day, but recovery rates of the three main compounds—(*E*)-2-hexenal, (*E*)-2-hexenol, and (*E*)-2-hexenoic acid—strongly differed. Fescemyer and Mumma (1983) induced discharge of defensive prothoracic glands of the dytiscid beetles *Arabus seriatus* Say and *A. obtusatus* Say; the beetles did not recover full amounts of secretion during the study and the 3 compounds were recovered at very different rates, with a sigmoidal curve for deoxycorticosterone, a saturated curve for unknown I, and an unimodal curve for unknown II.

Except for *C. gigantea*, all investigated species recovered defensive secretions, at least to some amount, which further suggests that the findings of Raspotnig (2006) probably reflect some methodological artifact. The few available studies do not allow general conclusions on the dynamics of secretion regeneration in arthropods, but the combination of quantification and predation tests might shed light on the adaptive values of costs invested in allomone restoration.

Predation Tests Until this study, no investigation of defensive secretion regeneration has quantified the level of post-depletion restoration that is needed for chemical defense in an arthropod. Herein, successful predation probably occurred mostly in those mite individuals that had not recovered significant amounts of defensive allomones. The broad range of absolute amounts of regenerated secretions during the experimental time course is known also from recovery studies in insects (Fescemyer and Mumma, 1983; Baldwin et al., 1990), and does not allow precise quantification of the

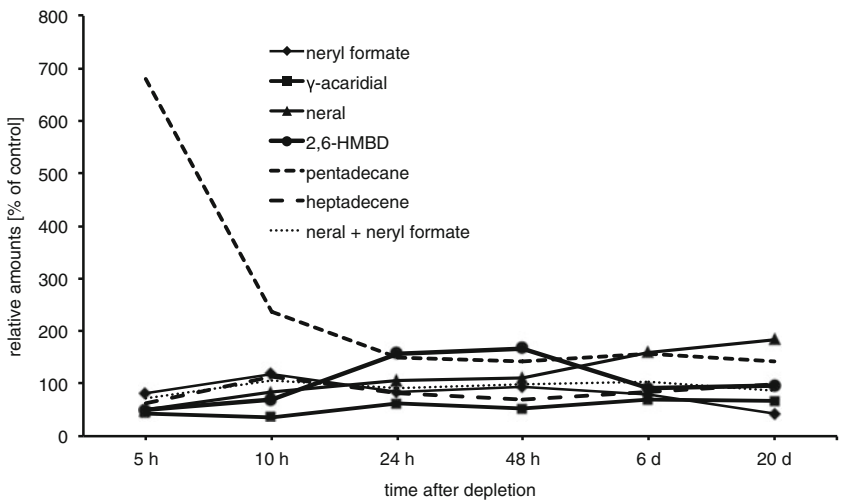

Fig. 6 Recovery of main compounds from the oil-gland secretion of *Archegozetes longisetosus*, compared to the relative amounts in the control group. Each point represents the average of 10 replicates

repellency threshold, but it should be between 100 % of MHO (max. amount after 24 hr) to 200 % of MHO (max. amount after 48 hr). While the latter amount is in the range of the control group, a comparison of the averages is probably more conservative, although biased by those specimens having not recovered any secretions. Using this approach, a refilling of the glands to about 25 % of the original amount (corresponding to 105 % of MHO at 48 hr vs. 451 % of MHO in the control) seems to be the minimum needed for effective chemical defense, at least against a large predator like *Stenus*. This seems plausible when taking into account the investment of resources in the regeneration of secretions: the synthetic rate was 25 times higher in the first 48 hr, indicating the biological importance to recover this minimum effective amount of secretion. Raspotnig (2006) reported that complete oil-gland depletion required no more than five mechanical stimulations in *C. gigantea*, which would be also in the range of 20–25 % of oil-gland secretions for a single defensive reaction.

While *Stenus juno* is a large model predator, in their natural habitats oribatid mites probably more frequently have to face smaller predators, with a lower mechanical impact on their prey (Peschel et al., 2006). The importance of chemical defense against such predators and the specific threshold secretion amounts remain to be uncovered, but such allomones almost certainly are at least partly responsible for the 'enemy-free-space' that non-astigmatid oribatid mites seem to live in (Peschel et al., 2006; Heethoff et al., 2011b; Heethoff and Raspotnig, 2012a). The high initial investment in regeneration of defensive secretions provides the first evidence that each individual pays a high and permanent cost for adaptively significant predator defense, and that the 'enemy-free-space' is not 'free' to acquire.

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Defensive Compounds and Male-Produced Sex Pheromone of the Stink Bug, *Agroecus griseus*

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Abstract *Agroecus griseus* is a serious corn pest in Brazil. Contents of the dorsal abdominal glands (DAGs) in nymphs, and the metathoracic gland (MTG) in adults of this species were characterized and quantified. Compounds found were similar to those of other Pentatomidae species and included aliphatic hydrocarbons, aldehydes, oxoalkenals, and esters. However, two compounds were found in the MTG that have not been described previously for this family. Mass spectrometry, infrared spectroscopy, and gas chromatographic analysis using coinjection with authentic standards confirmed the identities of the compounds as enantiopure (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate. The five nymphal instars showed significantly different ratios of components, mainly between those of the first and later instars. No significant differences were detected in MTG contents between sexes. Gas chromatography (GC) analysis of aeration extracts of males and females showed the presence of a compound released exclusively by males. Gas chromatography – electroantennographic detection (GC-EAD) assays indicated that the male-specific compound is bioactive in females, suggesting the presence of an attractant pheromone. The mass spectrum and infrared data for this compound matched with methyl 2,6,10-trimethyltridecanoate, a sex pheromone component previously detected in the stink bugs, *Euschistus heros* and *E. obscurus*. The synthetic standard coeluted with the natural pheromone on two different GC stationary phases, confirming the proposed structure. Y-tube olfactometer assays showed that the synthetic standard was strongly attractive to females, and GC-EAD tests produced responses with antennae from females similar to those of the natural pheromone.

Keywords GC-FTIR · Heteroptera Pentatomidae · Dorsal abdominal gland · Metathoracic gland · (*S*)-2-methylbutyl acetate · Methyl 2,6,10-trimethyltridecanoate · Agricultural pest

Introduction

Stink bugs (Heteroptera: Pentatomidae) are one of the main agricultural pests in the world, and they have become increasingly problematic with the advent of genetically modified crops (Bundy and McPherson, 2000). These pests produce large quantities of strong-smelling and irritating defense chemicals (allomones), which are released when the bugs are disturbed, serving as an alarm pheromone as well as a defense against predators (Aldrich, 1988).

Heteropteran nymphs produce exocrine secretions from dorsal abdominal glands (DAGs), the contents of which are shed along with the exuviae each time the nymph molts. Extraction of exuviae is a convenient method to obtain compounds made in DAGs for their identification (Borges and Aldrich, 1992). In adults, allomones are produced in the metathoracic scent gland (MTG). The identification of the contents of MTG secretions has received considerable attention, partly because these secretions constitute such an obvious defense, and because the large quantities of simple compounds produced are easy to analyze and identify (Aldrich, 1988; Ho and Millar, 2001; Fávaro et al., 2011).

The composition of stink bug allomones is similar for most species and includes hydrocarbons, as well as saturated and unsaturated aldehydes and esters (Aldrich, 1988). It is well-known that the proportions of compounds present in DAG secretions differ among the five nymphal instars

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(Borges and Aldrich, 1992; Fávaro et al., 2011; Fávaro and Zarbin, 2012). However, it has only recently been reported that the composition of the pentatomid MTG secretions may also differ according to the insect adult age (Fávaro et al., 2011; Fávaro and Zarbin, 2012).

Unlike defensive compounds, attractant pheromones of many stink bugs are produced in clusters of unicellular glands that are difficult to isolate by dissection and extraction (Millar, 2005). Thus, the most appropriate method for extraction is by aeration of live insects (Zarbin et al., 1999). In contrast to lepidopterans, pheromones identified to date for Pentatomidae exhibit a wide variety of chemical structures (Moraes et al., 2008). Pentatomid pheromones that have been identified include long-chain saturated hydrocarbons [*Phthia picta,* (Soldi et al., 2012)], terpenes [*Piezodorus hybneri,* (Leal et al., 1998) and *Nezara viridula,* (Aldrich et al., 1989)], and methyl esters [*Euschistus heros,* (Borges et al., 1998, 1999) and *Edessa meditabunda*, (Zarbin et al., 2012)].

Agroecus griseus Dallas (1851) is a major corn pests found in Brazil. These pests feed at the base of the plants, affecting tissue formation at the growth stage (Gassen, 1996). Besides *A. griseus*, the genus *Agroecus* contains another five described species (*A. brevicornis*, *A. lizerianus*, *A. scabricornis*, *A. ecuadoriensis,* and *A. reticultus*) that have been reported in several South America countries (Rider and Rolston, 1987). Although these insects are well-known, this work constitutes the first study of semiochemicals of *Agroecus* species.

The present work sought to: (a) identify and quantify nymphal exocrine compounds from the exuviae of the five nymphal instars; (b) identify and quantify the chemical components of the MTG in adults; and (c) identify the sex pheromone(s) of *A. griseus* by artificially testing the attractiveness of synthetic pheromone.

Methods and Materials

Insects Nymphs and adults of *A. griseus* were collected at EMBRAPA soybean fields in Londrina, Paraná State, Brazil (23º11' S, 51º11' W). Adults were sexed and separated from nymphs, and insects were maintained in plastic cages (35 x 20 x 20 cm) at 26±2 °C with 70 % relative humidity, and a 14/10 h L/D photoperiod. The colony was reared on soybean seeds (*Glycine max*), green beans (*Phaseolus vulgaris*), peanuts (*Arachis hypogaea*), and glossy privet fruits (*Ligustrum lucidum*). The food was replaced every 3 d.

Extraction of Dorsal Abdominal Glands Contents Hexane extracts of DAGs of the five instars were prepared by suspending exuviae in analytical-grade hexane after being collected≤24 h after ecdysis. Concentrations of the extracts were three exuviae per 100 µl for the first instar, one exuviae per 100 µl for the second and third instars, one exuviae per 200 µl for the fourth instar, and one exuviae per 400 µl for the fifth instar. Exuviae were extracted for 24 h at room temperature, after which the hexane extracts were transferred to another clean vial and stored at -20 °C until analysis. Three extracts were prepared and analyzed for each nymphal instar.

Extraction of Metathoracic Gland Contents A 30-d-old *A. griseus* adult was pinned dorsal side up through the prothorax in a Petri dish and submerged in tap water. The dissection process (using small surgical scissors and sharpened forceps) consisted of removing the wings, cutting the lateral margins of the abdomen anteriorly up to the metathorax, and transversely cutting the anterior margin of the scutellum. The tergal cuticle was pulled back, and the viscera were removed. The scent gland complex, located in the ventral metathoracic region, could subsequently be reached and removed by cutting laterally through the meso- and metathorax, turning the preparation over, and cutting transversely between the meta- and prothorax. The gland reservoir, including the lateral accessory glands (Aldrich et al., 1978), was removed, dried with tissue paper, immersed in 1000 µl of analytical grade hexane, and stored at -20 °C until being analyzed.

Collection of Volatile Compounds Volatile compounds were collected by the aeration method (Zarbin et al., 1999). Groups of 5 males and females were separately placed in glass chambers (25 x 9 cm ID) containing privet fruits. The collecting apparatus was maintained at the same temperature and photoperiod as the colony. A continuous 1 L/min flow of humidified and charcoal-filtered air was pulled through each chamber, drawing the volatile compounds through glass traps (11 x 1 cm ID) containing 60 mg of Super-Q adsorbent polymer (Alltech Associates Inc., Deerfield, IL, USA). Adsorbed volatile compounds were eluted from the polymer once a day with doubly distilled hexane (2 ml), concentrated with argon to 50 µl (10 µL per insect), and stored at -20 °C until analysis.

Chemical Analysis Volatile extracts were analyzed with a Shimadzu GC2010 gas chromatograph (GC) equipped with a FID detector, RTX-5 (Restek Bellefonte, PA, USA; 30 m x 0.25 mm x 0.25 µm film thickness) capillary column and helium as carrier gas. The GC was operated in splitless mode (250 °C). The temperature profile for gland extract analyses began at 50 °C for 1 min, increasing at 7 °C/min until reaching 250 °C, and maintaining this temperature for 10 min. Aeration extract analyses were conducted by starting the temperature profile at 100 °C for 1 min, increasing at 7 °C/min until reaching 250 °C, and holding at this temperature for 10 min. To determine the Kovats indices (Lubeck

and Sutton, 1983) and to examine the coinjection of the natural products with synthetic standards, RTX-WAX (Restek, 30 m x 0.25 mm x 0.25 μm film thickness) and EC™-1 (Alltech Associates, Inc., Deerfield, IL, USA; 30 m x 0.25 mm x 0.25 μm film thickness) capillary columns were employed. Chiral separations were obtained on a β-DEX™ 325 capillary column [25 % 2,3-di-O-methyl-6-O-TBDMS-β-cyclodextrin embedded in SPB-20 poly (20 % phenyl/80 % dimethylsiloxane), 30 m x 0.25 mm x 0.25 μm ID; Supelco, Bellefonte, PA, USA] using helium as carrier gas (1 ml/min) at 40 °C.

Gas chromatography-mass spectrometry (GC-MS) data were acquired using a Shimadzu QP2010-Plus electron ionization mass detector operating in the electron impact mode (70 eV) with an RTX-5 (Restek, 30 m x 0.25 mm x 0.25 μm) capillary column. The injector mode and program temperature were the same as described above. The National Institute of Standards and Technology (NIST) mass spectra chemical database was used.

Extracts also were analyzed by GC-Fourier transform infrared spectroscopy (GC-FTIR) with a Shimadzu GC2010 GC coupled to a DiscovIR-GC infrared detector (4000-750 cm^{-1}, resolution of 8 cm^{-1}; Spectra Analysis, Marlborough, MS, USA). The capillary column, injector mode and program temperature were the same as those previously described.

Gas chromatography-electroantennographic detection (GC-EAD) analysis data were acquired with a Shimadzu GC2010 coupled to a Syntech® electroantennographic detector (Hilversum, Netherlands). The GC was equipped with an RTX-5 capillary column (Restek, 30 m x 0.25 mm x 0.25 μm), operated in splitless mode (250 °C), and programmed to run at 100 °C for 1 min and subsequently increase by 7 °C/min to 250 °C. The antennae were fixed between the stainless steel electrodes using conductive gel (Signa gel, Parker Laboratories, Inc., Fairfield, NJ, USA). The electroantennogram were recorded on the Syntech GC-EAD32 program (version 4.6).

Micro-Derivatization

Hydrogenation with Palladium on Charcoal (Pd/C) To a hexane MTG extract in a glass vial~0.5 mg of Pd/C (5 % Pd) were added. A balloon filled with hydrogen was attached to the vial, and the reaction was stirred for approximately 3 h. The product solution was filtered and analyzed by GC (Attygalle, 1998).

Chemical Standards (*E*)-2-Hexen-1-ol, (*E*)-2-octenal, and (*E*)-2-decen-1-ol were purchased from Acros Organics (Geel, Turnhout, Belgium). Undecane, dodecane, tridecane, 1-tridecene, and 1-tetradecanol were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). (*E*)-4-Oxo-2-hexenal and (*E*)-4-oxo-2-decenal were gifts from Dr. K. Chauhan of the USDA-ARS (Beltsville, MD, USA) (Feldlaufer et al., 2010), and Dr. J. G. Millar of the University of California Riverside (CA, USA) (Moreira and Millar, 2005), respectively.

(*S*)-2-Methylbutyl, 3-methyl-2-butenyl, (*E*)-2-hexenyl, and (*E*)-2-octenyl acetates were prepared from their respective precursor alcohols (Ho and Millar, 2001; Fucarino et al., 2004). Additionally, 3-methylbutyl acetate was made via the hydrogenation of 3-methyl-2-butenyl acetate over Pd/C (5 % Pd) at room temperature and 20 psi H_2 in a Parr apparatus. The aldehydes (*E*)-2-hexenal, (*E*)-2-decenal, and tetradecanal were made by the Ferreira and Zarbin (1996) method using a mixture of their precursor alcohols and PCC. Methyl 2,6,10-trimethyltridecanoate was previously synthesized by Zarbin et al. (2000).

Olfactometer Bioassays with Synthetic Sex Pheromone Behavioral responses of *A. griseus* to the synthetic pheromone were tested in a Y-tube olfactometer using humidified, charcoal-filtered air flowing at 2.5 L/min. The olfactometer consisted of a Y-shaped glass tube (4 x 40 cm) with two 20 cm arms. The odorant source was placed at the end of the arms, consisting of a piece of filter paper (2 x 2 cm) impregnated

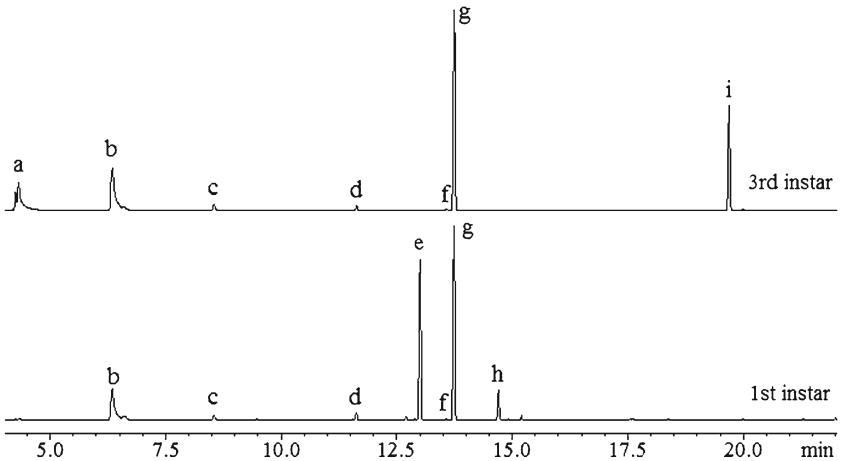

Fig. 1 Gas chromatogram of exuvial extracts from the first- and third-instar nymphs of *Agroecus griseus*. Compounds: (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, (*E*)-2-decenal **(e)**, 1-tridecene **(f)**, tridecane **(g)**, (*E*)-4-oxo-2-decenal **(h),** and tetradecanal **(i)**

Table 1 Relative abundances (%) (mean±SD) of compounds present in *Agroecus griseus* exuvial extracts from first to fifth-instars. Tukey test, $P<0.05$

Compound		1st	2nd	3rd	4th	5th
a	(*E*)-2-Hexenal[a]	0a	15.9±0.7b	13.9±1.3b	16.5±1.9b	14.0±0.3b
b	(*E*)-4-Oxo-2-hexenal[a]	18.2±1.8a	18.3±2.8a	10.7±2.5b	15.9±9.0ab	3.9±1.9c
c	(*E*)-2-Octenal[a]	1.3±0.5a	1.4±0.1a	3.9±0.5ab	6.5±1.2b	7.5±1.5b
d	Dodecane[a]	1.1±0.4ab	0.9±0.1a	1.5±0.1ab	1.5±0.2ab	2.1±0.3b
e	(*E*)-2-Decenal[a]	31.8±2.2a	0b	0b	0b	0b
f	1-Tridecene	0.2±0.1a	0.4±0.1a	0.8±0.1a	0.7±0.0a	0.7±0.2a
g	Tridecane[a]	43.9±2.8a	43.0±1.9a	48.4±4.9ab	41.3±8.5a	63.3±2.9b
h	(*E*)-4-Oxo-2-decenal[a]	3.5±1.6a	0b	0b	0b	0b
i	Tetradecanal[a]	0a	20.2±1.1b	20.8±1.8b	17.6±4.0bc	8.6±5.5 ac

[a]Compounds that showed significant differences between the instars

with the synthetic compounds or hexane (control). Two experiments were conducted to test the biological activity of the synthetic male-specific compound by measuring the response of females to the compound vs. control, and the response of males to the same compound vs. control.

An insect was introduced into the base of the olfactometer, and its behavior was observed for 15 min. A positive response was defined as the insect walking against the airflow more than 5 cm into an arm toward the odor source and remaining there for more than 2 min. No response was defined as the insect not leaving the main tube. Each insect was counted as one data point, and was tested only once. The odor source was replaced after each test. Insects that did not choose either of the arms were excluded from statistical analysis. The olfactometer was moved after every three tests to cancel positional effects.

Statistical Analyses Analysis of variance (ANOVA) followed by the Tukey test was used to compare the percentages of compounds present in exuviae and MTG extracts. Response data of olfactometer bioassays with the synthetic male-specific compound were analyzed using a *Chi-square* test. All tests were performed using the BioEstat program (version 5.0) (Ayres et al., 2003).

Results and discussion

A maximum of nine compounds were found in secretions from the DAGs of *A. griseus* nymphs (Fig. 1). By comparing the mass spectra of these compounds with those for other stink bugs previously studied (Fávaro et al., 2011; Fávaro and Zarbin, 2012), followed by coinjection with synthetic standards, the nine abundantly encountered compounds were identified as (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, (*E*)-2-decenal **(e)**, 1-tridecene **(f)**, tridecane **(g)**, (*E*)-4-oxo-2-decenal **(h),** and tetradecanal **(i)**.

The GC trace for the exuvial extract of the first instar nymphs was significantly different from that for the second to fifth instars according to analysis of variance, while the composition of exuvial extracts for the second to fifth instars did not differ significantly (Table 1). Tridecane **(g)** and (*E*)-2-decenal **(e)** were detected as the major components from the first instar followed by lower amounts of (*E*)-4-oxo-2-hexenal **(b)** and (*E*)-4-oxo-2-decenal **(h)**. The major component from the second instar was tridecane **(g)** followed by significant amounts of (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b),** and tetradecanal **(i)**.

Almost all compounds varied in relative abundances among the different exuvial extracts (Table 1). However, the main difference was the appearance of two compounds only in the first instar, specifically, (*E*)-2-decenal **(e)** and (*E*)-4-oxo-2-decenal **(h)**, and the absence of two others in this instar, specifically, (*E*)-2-hexenal **(a)** and tetradecanal **(i)**. Other studies of DAG secretory compounds showed that first-instars of some, but not all, pentatomid species contain (*E*)-4-oxo-2-decenal, which is totally absent in the secretions of later instars (Borges and Aldrich, 1992). (*E*)-4-Oxo-2-decenal mediates the aggregation behavior of first-instars

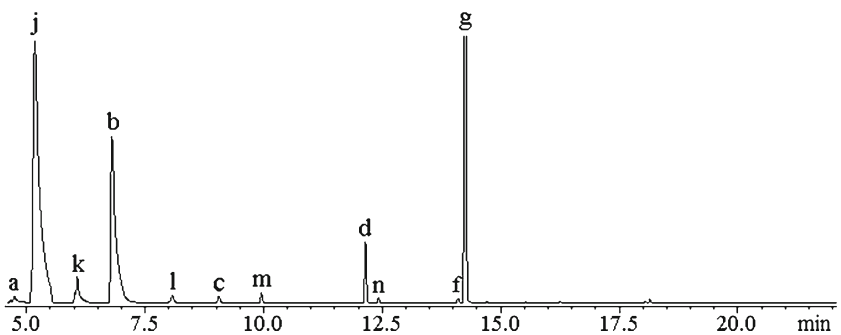

Fig. 2 Gas chromatogram of an MTG extract of an *Agroecus griseus* adult. Compounds: (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, 1-tridecene **(f)**, tridecane **(g)**, (*S*)-2-methylbutyl acetate **(j)**, 3-methyl-2-butenyl acetate **(k)**, (*E*)-2-hexenyl acetate **(l)**, undecane **(m),** and (*E*)-2-octenyl acetate **(n)**

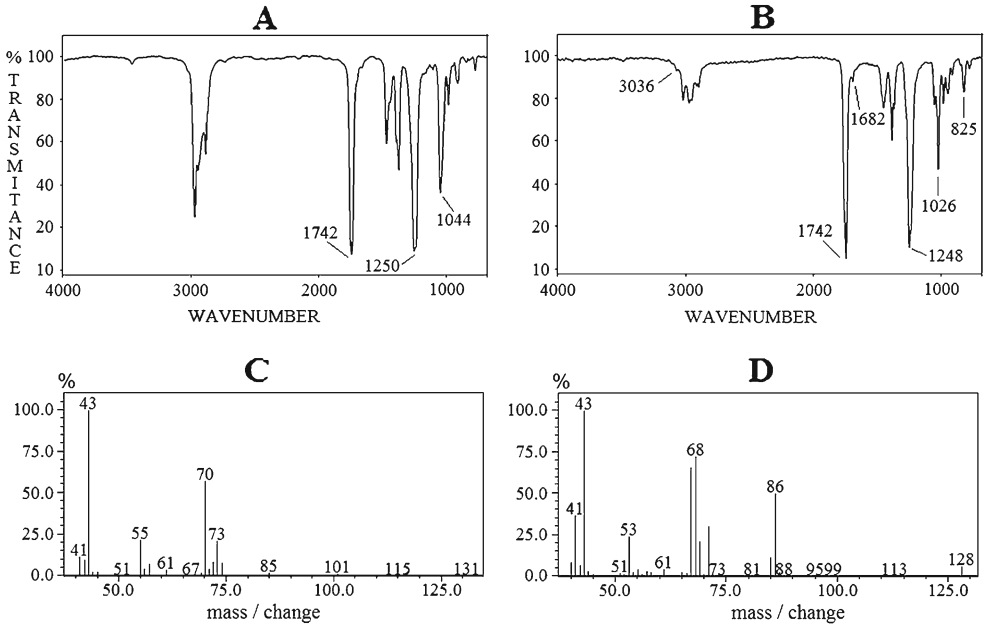

Fig. 3 Infrared and mass spectra of compounds **j** (**A** and **C**) **k** (**B** and **D**)

(Pavis et al., 1994; Fucarino et al., 2004), behavior that is strongly expressed by *A. griseus* nymphs.

As in other stink bugs, the MTG of *A. griseus* adults is well-developed with an orange-colored reservoir easily recognizable upon dissection (Aldrich, 1988). Analysis of the MTG extract showed the same compounds were present as found in late-instars of the *A. griseus* nymphs, with the exception of tetradecanal **(i)**, along with five new components (**j-n**, Fig. 2).

Three *A. griseus* MTG components exhibited mass spectra that matched those from other stink bugs we have recently studied (Fávaro et al., 2011; Fávaro and Zarbin, 2012), and these compounds were confirmed by GC coinjection with synthetic standards as (*E*)-2-hexenyl acetate **(l)**, undecane **(m),** and (*E*)-2-octenyl acetate (**n**). However, matching mass spectra for compounds **j** and **k** were not found, and their structures were identified by a combination of GC-MS and GC-FTIR, as well as micro-derivatization.

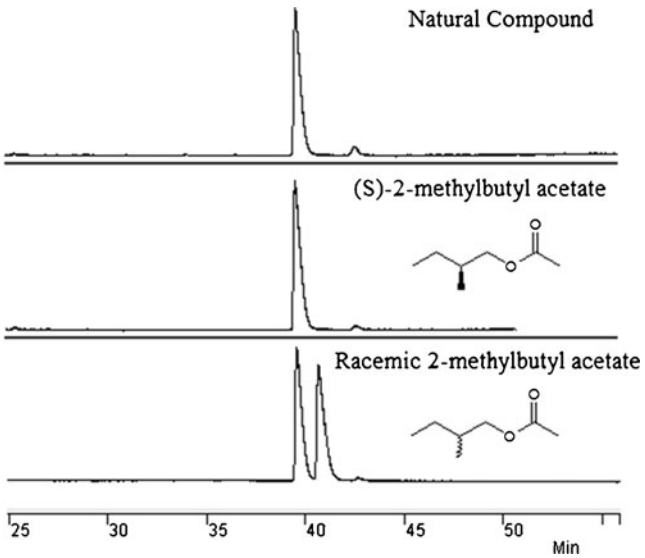

Fig. 4 Resolution of 2-methylbuthyl acetate (**j**) enantiomers on a cyclodextrin-based column

The GC-FTIR spectrum of compound **j** (Fig. 3A) exhibits three characteristic ester stretching frequencies at 1044 cm^{-1}, 1250 cm^{-1}, and 1742 cm^{-1}, which are due to O-C-C, C-O-C, and C=O stretching, respectively (Smith, 1999). The mass spectrum of this compound (Fig. 3C) showed a molecular ion ($M^+ + 1$) at *m/z* 131, a fragment at *m/z* 70, due to the loss of an acetic acid molecule ($M^+ - 60$) characteristic of acetate compounds, and a base peak at *m/z* 43 resulting from the cleavage of the C-O single bond of the ester (Silverstein et al., 2005). Comparison of the mass spectrum of this

Table 2 Amount (%) (mean±SD) of compounds present in the metathoracic gland (MTG) extracts of *Agroecus griseus* adults. Values in % by MTG

Compound		Mean (%)±SD
a	(*E*)-2-Hexenal	2.28±1.64
j	(*S*)-2-Methylbutyl acetate	30.28±2.46
k	3-Methyl-2-butenyl acetate	4.11±0.22
b	(*E*)-4-Oxo-2-hexenal	14.95±2.24
l	(*E*)-2-Hexenyl acetate	6.64±4.28
c	(*E*)-2-Octenal	0.52±0.02
m	Undecane	1.71±0.21
d	Dodecane	3.95±0.08
n	(*E*)-2-Octenyl acetate	0.70±0.20
f	1-Tridecene	1.42±0.06
g	Tridecane	33.48±1.40

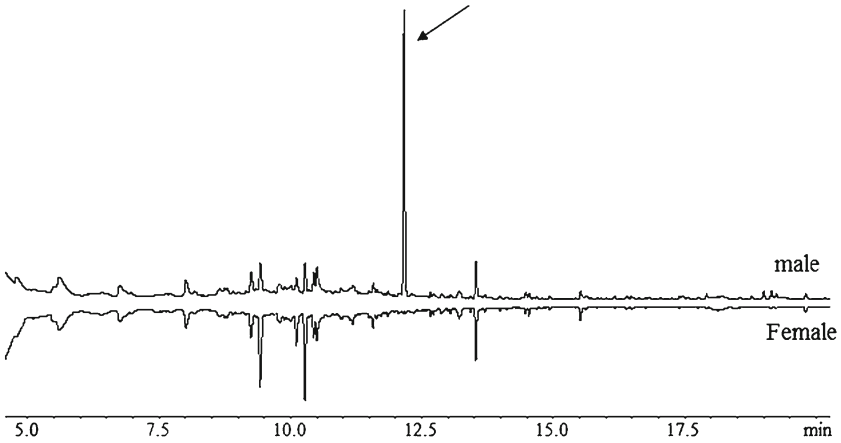

Fig. 5 Comparison of gas chromatograms of volatile compounds obtained from *Agroecus griseus* males and females showing the male-specific compound

compound with the NIST computer database, along with infrared data, suggested two possible chemical structures, specifically, 2- or 3-methylbutyl acetate. From the coinjection of the extract with the synthetic standards of these two acetates on three different columns (EC-1, RTX-5, and RTX-WAX), the identity of the compound **j** was determined to be 2-methylbutyl acetate.

To establish the absolute configuration of this molecule, GC analysis employing a chiral column was conducted. Racemic **j** was baseline resolved on a cyclodextrin-based capillary column and showed two peaks with retention times of 39.5 and 40.6 min (Fig. 4). The synthetic (*S*)-enantiomer co-eluted with the earlier eluting peak, and the purified natural product shared the same retention time as this compound. Therefore, the acetate **j** present in the *A. griseus* MTG content was fully characterized as enantiopure (*S*)-2-methylbutyl acetate.

Besides the ester absorbances (Fig. 3B) at 1026 cm^{-1} (O-C-C), 1248 cm^{-1} (C-C-O), and 1742 cm^{-1} (C=O), compound **k** presented three bands at 825 cm^{-1} (C-H bend), 1682 cm^{-1} (C=C stretch), and 3036 cm^{-1} (C-H stretch) that are characteristic of a non-terminal, trisubstituted alkene (Smith, 1999). In the mass spectrum of this compound (Fig. 3D), a base peak at *m/z 43*, and a molecular ion peak at *m/z* 128 (M^+) were observed. Another intense fragment at *m/z* 68 also was detected resulting from the loss of acetic acid (M^+ - 60) (Silverstein et al., 2005). From the data described above, and by comparison with the NIST mass spectra database, two different chemical structures were proposed: 2-methyl-2-butenyl acetate and 3-methyl-2-butenyl acetate.

To clarify further the structure of this compound, the purified natural product was hydrogenated over Pd/C, and the resulting product was co-injected on three different GC columns with authentic samples of 2-methylbutyl acetate and 3-methylbutyl acetate, which were available in our laboratory. These analyses unambiguously identified compound **k** as 3-methyl-2-butenyl acetate.

Thus, the MTG scent blend of *A. griseus* contains high concentrations of tridecane (**g**), which is also the major component in nymphs, (*S*)-2-methylbutyl acetate (**j**) and (*E*)-4-oxo-2-hexenal (**b**). All the other components appear only at trace levels (Table 2).

Most of the defensive compounds identified for *A. griseus* have been found previously for other Pentatomidae species (Moraes et al., 2008). However, our identification of 2-methylbutyl acetate and 3-methyl-2-butenyl acetate is the first time these compounds have been identified from the Pentatomidae. 2-Methylbutyl acetate also is found as a volatile plant component of several Orchidaceae species and in certain Fabaceae. 3-Methyl-2-butenyl acetate has been reported as a defensive compound only in a Hemiptera species, *Spilostethus rivulari* (Lygaeidae) (Staddon et al., 1985; El-Sayed, 2011).

Besides defensive compounds, attractant pheromones also can be produced in and released from the MTGs of

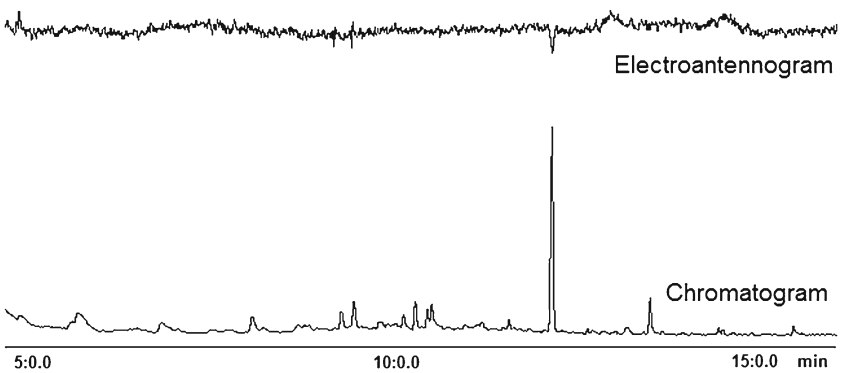

Fig. 6 Response of an antenna from an *Agroecus griseus* female to the male-specific compound released by conspecific males

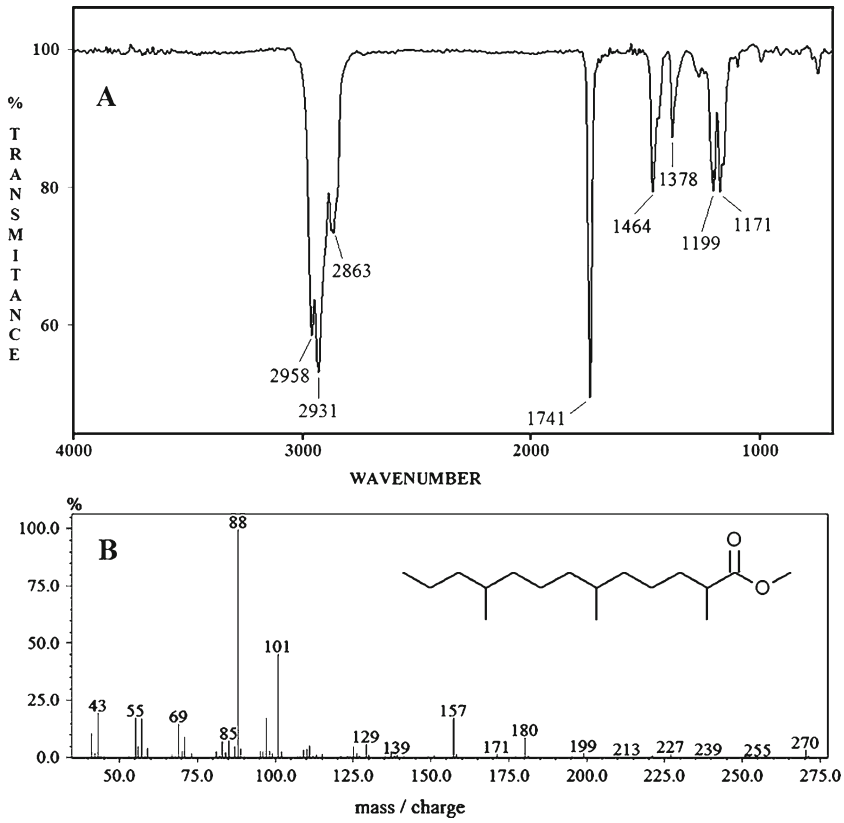

Fig. 7 Infrared (**A**) and electron impact mass spectra (**B**) of the *Agroecus griseus* male-specific compound

certain Heteroptera (Millar, 2005), probably from the lateral accessory glands attached to the MTG reservoir (Aldrich et al., 1997, 1999, 2000). MTG-derived attractant pheromones can be produced by either males, as in some Lygaeidae and Alydidae (Leal et al., 1995; Aldrich et al., 1997,1999), or females, as in Miridae and some Alydidae (Smith et al., 1991; Leal et al., 1996; Millar et al., 1997; Aldrich et al., 2000; Zhang and Aldrich, 2003, 2008). We recently described that males of *P. picta* produce a specific sex pheromone in the lateral accessory glands (Soldi et al., 2012). However, no difference was observed between the MTG secretions of *A. griseus* males and females; therefore, we decided to use the aeration method to study the sex pheromone system of the species (Zarbin et al., 1999).

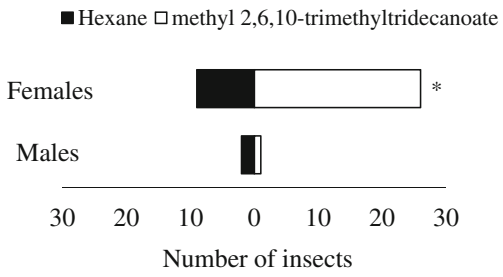

Fig. 8 Total number of *Agroecus griseus* males and females attracted to the synthetic compound, methyl 2,6,10-trimethyltridecanoate, in a Y-tube olfactometer. *Chi-square* test, $P<0.001$

Comparison of the gas chromatograms of aeration extracts collected from male and female *A. griseus* adults showed the presence of a male-specific compound, along with other compounds that were common for both sexes (Fig. 5). The Kovats index from this compound was calculated on two different columns: the RTX-WAX, which showed a value of 1889, and the RTX-5, with a value of 1752 being observed.

Electrophysiology analysis of the male volatile extract on the antennae of males and females was performed to determine whether the male-specific compound or other compounds in the extract showed biological activity for this species. Only antennae of females responded to the male-specific compound (Fig. 6), suggesting a sex pheromone mediating the behavior of *A. griseus*.

For the structural elucidation of the *A. griseus* sex pheromone, the male volatile extracts were analyzed by GC-MS and GC-FTIR. The GC-FTIR spectrum (Fig. 7A) showed a band at 1741 cm^{-1}, which is consistent with the C=O stretching of esters, and two other bands at 1171 cm^{-1} and 1199 cm^{-1}, corresponding to the C-O-C stretching of this functional group (Smith, 1999). Among the main fragments present in the mass spectrum (Fig. 7B), a molecular ion of *m/z* 270, a base peak of *m/z* 88, and a second most intense peak at *m/z* 101 were observed, fragments characteristic of a methyl branch bonded to the α-carbon of a methyl ester. Two peaks at *m/z* 157 and *m/z* 227 showed a high relative

intensity, suggesting the presence of two other methyl branches (Silverstein et al., 2005). This same fragmentation pattern was described previously for methyl 2,6,10-trimethyltridecanoate, which was identified as a component of the sex pheromone of the stink bugs, *Euschistus heros* (Aldrich et al., 1991) and *E. obscurus* (Borges and Aldrich, 1994).

Several similarities between *Agroecus* and *Euschistus* had already been reported, but only morphologically (Rider and Rolston, 1987). It is common to find compounds that are repeated in the pheromonal mixture of related insect genera. For example, *cis* and *trans-Z*-bisabolene epoxides are present in the sex pheromone mixtures of two *Nezara* species (*N. viridula* and *N. antennata*), and four *Chinavia* species (formerly *Acrosternum,* Schwertner and Grazia, 2007; *A. aseadum*, *A. hilare*, *A. marginatum,* and *A. pennsylvanicum*) (Millar, 2005).

Methyl 2,6,10-trimethyltridecanoate was first synthesized by Mori and Murata (1994a), using a cyclopropane cleavage by a Julia rearrangement as the key reaction. The same authors later showed the synthesis of all of the eight possible stereoisomers of this compound (Mori and Murata, 1994b). More recently, Zarbin et al. (2000) described an alternative six-step synthetic route to this compound, starting from (±)-citronellol with a 16 % overall yield.

The synthetic compound (Zarbin et al., 2000) was co-injected with the male volatile compound extracts, and co-elution with the male-specific compound was observed on two different columns (RTX-5 and RTX-WAX). The mass and infrared spectra of the synthetic compound also were identical to those of the natural product, confirming methyl 2,6,10-trimethyltridecanoate as the male-specific compound of *A. griseus*.

The biological activity of the synthetic compound was analyzed with GC-EAD and behavioral tests using male and female *A. griseus* in a Y-shaped olfactometer. The synthetic compound elicited antennal responses in females but not in males, as did the natural compound. Methyl 2,6,10-trimethyltridecanoate was highly attractive to females in olfactometer bioassays when tested against the solvent control [insects used=40, response (N)=35, treatment 26 (74 %), control 9 (26 %), $P<0.001$], while the males show no such effect [insects used=10, response (N)=3, treatment 1 (33 %), control 2 (67 %)] (Fig. 8). These bioassay results implicate methyl 2,6,10-trimethyltridecanoate as a sex pheromone of *A. griseus*.

Methyl 2,6,10-trimethyltridecanoate has eight stereoisomers, and the importance of chirality on a compound's bioactivity as an insect pheromone has been demonstrated previously (Mori, 2007). Although the mixture of all the eight isomers of this pheromone was attractive toward *E. heros* females, the (2*R*,6*R*,10*S*)-isomer proved to be more effective than the stereoisomeric mixture in olfactometer bioassays (Costa et al., 2000). *Agroecus griseus* females also were attracted to a racemic mixture of the pheromone; however, testing whether they exhibit a similar increased attraction to the enantiopure compound has yet to be completed. Nor has the possibility that the unusual MTG esters, (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate, are involved in pheromonal attraction been eliminated.

In summary, this work describes all of the defense compounds present in the DAGs of nymphs and MTG of adults of *A. griseus*, as well as a sex pheromone produced by males of this species. Besides some typical defense compounds found in stink bugs, two components that have not been described previously for pentatomids also were discovered: (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate. A sex pheromone of *A. griseus* was identified as methyl 2,6,10-trimethyltridecanoate, and its biological activity was demonstrated by behavioral and EAD tests. Field experiments employing the synthetic molecules are now underway.

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Cuticular Hydrocarbons of the South American Fruit Fly *Anastrepha fraterculus*: Variability with Sex and Age

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Abstract Insect cuticular hydrocarbons are usually species-specific mixtures and may serve for species and gender recognition. They are, therefore, widely used in the chemotaxonomy and zoogeography of various insect taxa. In order to provide a basic study for further comparative analyses of cuticular hydrocarbon (CHC) profiles of cryptic species hidden within the South American fruit fly *Anastrepha fraterculus* complex (Diptera: Tephritidae), we analyzed the composition of the CHCs and their production with respect to age and sex in a laboratory population from Tucuman, Argentina. Several techniques of gas chromatography with mass spectrometric detection have been used in order to develop a suitable method for CHC identification, i.e., GC-MS in EI mode, GC-MS in CI mode, and GC×GC/TOFMS. Our analyses revealed a complex profile of aliphatic hydrocarbons in both males and females, consisting predominantly of *n*-alkanes, methyl-branched alkanes, as well as of alkenes and alkadienes. In young individuals (up to about 5 days after emergence), the CHC profiles were similar in males and females. However, in older flies, these profiles diverged and became clearly sex-specific. The temporal dynamics of the CHC patterns in both sexes were evaluated using multivariate exploratory techniques.

Keywords *Anastrepha fraterculus* species complex · Cuticular hydrocarbons · Sex-specific differences · Age-dependent production · Gas chromatography-mass spectrometry

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Introduction

Anastrepha (Diptera: Tephritidae) is the largest and most economically important genus of Tephritidae in most countries of North and South America (Hernández-Ortiz and Aluja, 1993; Steck, 1999; Zucchi, 2000, 2007). *Anastrepha* includes 197 currently recognized species, some of which, however, are now known to be cryptic species complexes (Aluja and Norrbom, 2001). Currently, one of the most studied groups is the *A. fraterculus* complex. The South American fruit fly, *A. fraterculus* (Wiedemann), is widespread, from Texas to Argentina, with two species occurring in the Antilles. It is a highly destructive pest that imposes quarantine restrictions for fruit export to many

countries (Hernández-Ortiz and Aluja, 1993; Steck, 1999; Zucchi, 2000, 2007; Aluja and Norrbom, 2001). The differences in morphology, genetics, and mating behavior observed among individual populations throughout the geographic range suggest that *A. fraterculus* is a complex of cryptic species rather than a single biological entity (Morgante et al., 1980; Malavasi and Morgante, 1982; Solferini and Morgante, 1987; Steck, 1991; Steck and Sheppard, 1993; Selivon, 1996; Hernandez et al., 2004). To disentangle this complex is a crucial task in order to understand better the ongoing speciation process within *Anastrepha* as well as to design efficient and appropriate techniques of biological control.

Cuticular hydrocarbons (CHCs) are nonpolar compounds present on the epicuticle of all insects. Their primary function is to protect the insect against desiccation. Secondarily, they play an important role in many species for intra- and interspecific communication in a plethora of diverse contexts, such as species and gender recognition, fertility signaling, chemical mimicry, and many other functions described especially in social insects. Thus, CHCs have become an excellent identification tool in various fields of insect biology (Blomquist and Bagnères, 2010; Kather and Martin, 2012). Qualitative and/or quantitative specificity related to sex and mating status has been demonstrated in a large list of insect species including numerous dipterans (see e.g., Howard and Blomquist, 2005). Although the lipids of several dipteran species are known to undergo age-related changes during the adult stage, additional studies are needed to link these changes directly in the genus *Anastrepha* to age classification by GC/MS (Perez-Mendoza et al., 2002). In previous studies on the family Tephritidae, the CHC pattern has been shown to provide a potential cue for the recognition of species, populations, and gender (Carlson and Yocom, 1986; Lavine et al., 1992; Goh et al., 1993; Sutton and Carlson, 1993; Sutton and Steck, 1994).

Despite previous studies, there is a piece of information missing in a precise chemical characterization of CHCs in the genus *Anastrepha*. The examination of the age- and gender-dependent changes of CHC profiles could reveal information for mate choice, i.e., the quality of the potential mate or its mating status. In this study, we describe the CHCs in laboratory females and males of *A. fraterculus* by using a set of gas chromatography-mass spectrometry (GC - MS) techniques. This knowledge is an essential basis for further studies, such as the analysis of possible differences in the CHC profiles of different species and forms of the *A. fraterculus* complex, which could be related to the potential role of CHCs as contact pheromones. We interpret our results with respect to the age of individuals by using multivariate statistics, because the age-grading of insects is important in the control and monitoring of both insect populations and vector-borne diseases (Reeves et al., 2010). Therefore, our results may serve as basis for fruit fly researchers to estimate the age of single-field specimens and will fill a gap in the missing knowledge regarding the age-dependent CHC production in *Anastrepha*.

Methods and Materials

Insect Origin Pupae of an *A. fraterculus* laboratory population (originally from Tucuman, Argentina) were obtained from the entomological laboratory FAO/IAEA (Seibersdorf, Austria). After emergence, insects were separated by sex and placed into plastic chambers (30×20.5×16 cm). Flies were fed an artificial diet composed of cane sugar and mineral water. The temperature of the insectarium was 25 °C, the relative humidity 60 %, and the photoperiod 14 hr.

Extraction of CHCs Starting from the day of emergence until the 30th d after emergence (d 0, 1, 2, 3, 5, 7, 14, 16, 20, 30), five male and five female flies were immobilized (−18 °C) and placed for 15 min into a desiccator to remove surface moisture. The CHCs were extracted individually with 0.5 ml hexane (Fluka, Germany) for 5 min in small glass vials. Two internal standards were used for quantification: methyl stearate (10 ng per 1 ml of the extract) and hexatriacontane (50 ng per 1 ml of the extract). Each extract was concentrated to approximately 100 μl by a constant flow of nitrogen, and stored in a freezer until analysis.

Chemical Analysis The identification and quantification of CHCs were performed on a gas chromatograph HP 6890 equipped with a ZB-5 ms capillary column (Phenomenex, USA; 30 m×250 μm i.d.×0.25 μm of the film of the stationary phase) coupled to a MASSPEC (MICROMASS, UK). The injector was operated in the splitless mode at 220 °C, and a helium flow rate of 1 ml/min and a transfer line temperature of 280 °C were used. The oven temperature program was as follows: 80 °C for 2 min, 15 °C/min to 300 °C, hold at 300 °C for 15 min. The data were analyzed using MassLynx (Version 4.0). The CHCs were quantified from chromatograms reconstructed from the *m/z* 57 ion; all of the hydrocarbons eluting after 6 min were integrated. For absolute quantification of the CHCs, the internal standard methyl stearate was used. To identify co-eluting peaks, two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/TOFMS) was applied. The GC×GC/TOFMS analyses were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with a non-moving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m×250 μm i.d.×0.25 μm film) was used for GC in the first dimension. The second-dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX,

USA; 2 m×100 μm i.d.×0.1 μm film). Helium was used as carrier gas at a constant flow of 1 ml/min. The temperature program for the primary GC oven was as follows: 80 °C for 2 min, then 80–300 °C at 10 °C/min, and finally a 10 min hold at 320 °C. The program in the secondary oven was 10 °C higher than in the primary one, and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration, and the cool time between the stages were set to 3.0, 0.4, and 1.1 sec, respectively. The transfer line to the TOFMS was operated at 260 °C. The source temperature was 250 °C with a filament bias voltage of −70 eV. The data-acquisition rate was 100 Hz (scans/sec) for the mass range of 29–400 amu. The detector voltage was 1750 V. For each sample, 1 μl was injected in the splitless mode. The inlet temperature was 200 °C. The purge time was 60 sec at a flow of 60 ml/min. Data were processed and consecutively visualized on 2D and 3D chromatograms using LECO ChromaTOF^{TM} software. A standard of *n*-alkanes (C_8–C_{36}; Sigma-Aldrich) was co-injected with authentic samples to determine the retention indices (RI) of the analytes. One RI value higher than 3600 was calculated by extrapolation using a data processing method (LECO ChromaTOF^{TM} software). The hydrocarbons were identified by a comparison of their MS fragmentation patterns and retention indices-RI (Kováts, 1965; Pomonis et al., 1980; Carlson and Yocom, 1986; Carlson et al., 1989, 1998; Lavine et al., 1992; Goh et al., 1993; Sutton and Carlson, 1993; Sutton and Steck, 1994; Geiselhardt et al. 2009). The double-bond positions of the monoenes and dienes were determined by interpreting the collision-induced mass spectra of the acetonitrile-formed radical cation *m/z* 54 adducts after chemical ionization (CI) and tandem mass spectrometry (MS/MS). The GC-CI/MS/MS were performed using a Varian 240MS ion-trap mass detector coupled to a Varian 450GC. All of the analyses were performed in the external ionization configuration. A VF-5 ms column (Varian Inc. Palo Alto, CA, USA; 30 m×250 μm i.d.×0.25 μm film) was used to separate the hydrocarbons. The temperature of the split GC injector was set to 250 °C, and the injector was operated in the splitless mode. The GC oven temperature was programmed as follows: 150 °C for 1 min, 5 °C/min to 300 °C and a hold for 5 min. Helium was used as carrier gas at a constant flow rate of 1 ml/min. The CI spectra were recorded using acetonitrile as the reagent gas with a mass range of *m/z* 60–600. The CI/MS/MS experiments of the alkene and alkadiene acetonitrile adducts $[M+54]^+$ were conducted and the mass spectra interpreted as described previously (Kroiss et al., 2011).

Statistics The patterns of the CHCs depending on age or sex were analyzed by multivariate methods (e.g., Kaltenpoth et al., 2007; Strohm et al., 2008). Peaks of the CHCs were integrated in MassLynx and exported. The relative peak areas as well as absolute amounts of all the substances were calculated based on the internal standards added to the samples before the GC/MS analysis. The absolute amounts of CHCs were compared across age groups for both males and females, respectively, using univariate ANOVA. For multivariate statistics, the relative peak areas were transformed to logcontrasts (Aitchison, 1986), and the number of variables was reduced by a principal component analysis (extracting the PCs with Eigenvalues >1). To test for differences in CHC profiles across age groups in males and females, we used several approaches. First, we performed hierarchical cluster analyses on the logcontrast-transformed relative peaks areas as well as on the extracted PCAs to assess the similarity of samples without *a priori* grouping. Furthermore, we conducted a discriminant analysis (DA) with males and females based on the PCAs. Finally, we performed multivariate analyses of variance (MANOVA) with the logcontrast-transformed relative peaks for males and females, respectively, to assess differences in individual compounds across age groups. All of the statistical tests were done using SPSS17.0.

Results

Analysis of Anastrepha CHCs A comparison of the data obtained by GC-CI/MS/MS, GC-EI/MS and GC×GC/TOFMS allowed for the characterization of 66 peaks (Table 1). The chain-length of the carbon backbones ranged from C_{13} to C_{37}. The hydrocarbon profiles of the males and females included 14 *n*-alkanes, 20 monomethyl alkanes, 2 dimethyl alkanes, 10 alkenes, 12 alkadienes, squalene, and 9 unidentified compounds. The most prominent peaks in all of the chromatograms were 2-methylnonacosane (2-MeC_{28}), 9-nonacosene (9-$C_{29:1}$), nonacosane (C_{29}), 8,14-hentriacontadiene (8,14-$C_{31:2}$), 11,14-hentriacontadiene (11,14-$C_{31:2}$), 9,16-pentatriacontadiene (9,16-$C_{35:2}$), and 8,16-pentatriacontadiene (8,16-$C_{35:2}$) (Figs. 1 and 2). For the identification of the double-bond position in the alkenes and alkadienes, we used CI/MS/MS with acetonitrile as the reagent gas. The typical fragment ions of the acetonitrile-derived adducts $[M+54]^+$ with monoenes and dienes are shown in Table 2.

Gender-Specific Differences in the CHC Composition The CHC profiles of the virgin males and females differed quantitatively and qualitatively. In mature males (<5 days), we identified a set of 7-monoenes that are absent from the females and immature males, i.e., 7-$C_{21:1}$, 7-$C_{22:1}$, 7-$C_{23:1}$, 7-$C_{25:1}$,and 7-$C_{33:1}$ (Table 1, Figs. 1 and 2). By contrast, we did not detect any sex-specific CHCs in the females. Significantly different patterns of CHCs were detected in both genders when running DA based on nine principal components (containing 81.9 % of the total variance)

Table 1 List of the cuticular hydrocarbons identified in ten different age groups of *Anastrepha fraterculus* males and females

No.	Hydrocarbon	RI[c]	Female										Male									
			day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30	day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30
1[a]	3-MeC$_{12}$	1275	-	-	-	-	++	++	++	++	++	-	-	-	-	-	++	++	++	++	++	-
2	*Unknown 1*	1322	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
3	3,7-DiMeC$_{14}$	1406	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
4	*Unknown 2*	1488	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
5[a]	9-MeC$_{15}$	1534	-	-	-	-	+	+	+	++	++	-	-	-	-	-	+	+	+	++	++	-
6	*Unknown 3*	1535	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
7[a]	4-MeC$_{15}$	1556	-	-	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+	++	++	-
8[a]	*n*-C$_{16}$	1600	-	-	++	++	++	++	++	++	++	-	-	-	++	++	++	++	++	++	++	-
9[a]	*n*-C$_{17}$	1700	-	-	++	++	++	++	++	++	++	+	-	-	+	+	+	+	+	++	++	+
10[a]	7-MeC$_{17}$	1744	+	+	++	++	+	+	+	++	++	+	+	++	++	+	+	+	++	++	++	+
11[a]	2-MeC$_{17}$	1764	+	+	+	+	+	++	++	+	++	+	+	+	+	++	+	++	+	++	++	-
12[a]	*n*-C$_{18}$	1800	+	+	+	++	+	+	+	++	++	+	+	+	+	++	+	+	+	++	++	+
13	*Unknown 4*	1810	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
14	*Unknown 5*	1917	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
15[a]	2-MeC$_{19}$	1961	+	+	+	+	+	+	+	++	++	-	+	+	+	++	+	+	++	++	++	-
16[a]	3-MeC$_{19}$	1978	+	+	+	+	+	++	++	tr	++	-	+	+	+	++	+	+	+	++	++	++
17[a]	*n*-C$_{20}$	2000	+	+	+	++	+	+	+	++	++	++	+	+	+	++	+	+	+	++	++	+
18[a,b]	7-C$_{21:1}$	2083	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	++	+++	+++
19[a]	*n*-C$_{21}$	2100	+	+	+	+	+	+	+	tr	+	++	+	+	+	+	+	+	++	++	++	++
20[a]	2-MeC$_{21}$	2165	+	+	++	+	+	+	+	++	++	++	+	+	+	++	+	+	+	++	++	++
21	3-MeC$_{21}$	2182	+	+	++	+	+	+	+	++	++	++	+	+	+	++	+	+	+	++	++	++
22[a,b]	7-C$_{22:1}$	2182	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	++	++
23[a]	*n*-C$_{22}$	2200	+	+	+	+	+	+	+	++	+	+	+	+	+	++	+	+	+	++	++	+
24[a,b]	7-C$_{23:1}$	2283	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+++	+++	+++	++
25[a]	*n*-C$_{23}$	2300	+	+	+	+	+	+	+	tr	+	+	+	+	+	++	+	+	++	++	++	++
26[a]	9-MeC$_{23}$	2336	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	++
27[a]	3-MeC$_{23}$	2375	+	+	+	++	+	+	++	+	+	+	+	+	+	++	+	+	+	++	+	+
28[a]	*n*-C$_{24}$	2400	+	+	+	++	+	+	+	++	+	+	+	+	+	+	+	+	+	+	++	++
29[a]	3,*y*-DiMeC$_{24}$	2406	+	+	+	++	+	+	+	++	+	+	+	+	+	+	+	+	+	+	++	++
30[a,b]	7-C$_{25:1}$	2483	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+	+	++
31[a]	*n*-C$_{25}$	2500	+	+	++	++	+	+	+	tr	+	+	+	+	+	++	+	++	+	++	+	++
32[a]	*n*-C$_{26}$	2600	+	+	++	++	+	tr	+	+	+	++	+	+	++	++	+	++	+	+	+	++
33[a]	2-MeC$_{26}$	2661	+	+	++	++	+	+	+	+	+	++	+	+	+	++	+	++	++	+	++	++

Table 1 (continued)

No.	Hydrocarbon	RI[c]	Female										Male									
			day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30	day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30
34[a]	*n*-C_{27}	2700	+	+	+	+	+	+	+	+	+	++	+	+	++	+	+	++	++	++	++	++
35	*Unknown 6*	2761	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
36	3-MeC_{27}	2775	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
37	*n*-C_{28}	2800	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
38[a]	Squalene	2823	+	+	+	+	+	+	tr	++	++	++	+	++	+	++	++	++	++	++	+	++
39	*Unknown 7*	2843	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
40[a]	2-MeC_{28}	2866	++	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +
41[a]	9-$C_{29:1}$	2883	++	+++	+++ +	+++ +	+++	+++	+++	+++ +	+++	+++	+++	+++ +	+++	+++ +	++	++	++	++	++	+++ +
42[a]	*n*-C_{29}	2900	++	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++	+++	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +	+++	+++	++	++
43[a]	13-/11-/9-MeC_{29}	2935	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	+	++	++
44[a]	7-MeC_{29}	2943	++	++	++	+	++	+	+	++	++	++	+	++	++	++	+	++	++	+	++	++
45[a]	3-MeC_{29}	2973	+	+	++	++	++	++	++	++	++	++	+	+	+	+	++	++	++	++	++	++
46[a]	2-MeC_{30}	3066	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++
47[a]	9,11-$C_{31:2}$	3067	++	+++	++	+++	+++	+++	+++	++	++	++	+++	++	+++	+++	+++ +	+++ +	+++	++	++	++
48[a]	8,14-$C_{31:2}$	3067	++	++	++	++	++	+++	+++	++	+++	++	++	++	++	++	++	+++	++	+++	++	++
49[a]	11,14-$C_{31:2}$	3067	+	+	+	+	+	++	++	++	++	++	+	+	++	+	+	tr	+	+	+	++
50	11-$C_{31:1}$	3081	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
51[a]	10-$C_{31:1}$	3081	+	+	+	+	+++	++	+	++	++	++	++	++	+++	+	+	+	++	++	++	++
52[a]	*n*-C_{31}	3100	+	+	+	+	+	++	++	++	++	++	+	+	++	+	+	tr	+	+	+	++
53	*Unknown 8*	3182	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
54[a]	9,15-$C_{33:2}$	3260	+	+	+	+	+++	++	+	++	++	++	++	++	+++	+	+	+	++	++	++	++
55[a]	9,16-$C_{33:2}$	3266	+	++	+	+	+	+	++	+	+	++	+++	++	+	++	+	+	+	tr	++	++
56[a]	9,17-$C_{33:2}$	3268	+	+	+	+	+++	++	+	++	++	++	++	++	+++	+	+	+	++	++	++	++
57[a]	7,13-$C_{33:2}$	3269	+	++	+	+	+	+	++	+	+	++	+++	++	+	++	+	+	+	tr	++	++
58[a]	7,19-$C_{33:2}$	3269	+	+	+	+	+++	++	+	++	++	++	++	++	+++	+	+	+	++	++	++	++
59[a]	11-$C_{33:1}$	3280	-	++	++	++	+	+	-	-	-	-	++	++	++	++	++	++	+	+	-	-
60[a,b]	7-$C_{33:1}$	3283	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-
61	*Unknown 9*	3308	-	-	-	tr	tr	tr	tr	tr	tr	tr	-	-	-	tr	tr	tr	tr	tr	tr	tr
62[a]	9,16-$C_{35:2}$	3466	++	++	++	++	++	++	++	++	+	+	++	++	++	+++	+++	++	++	++	++	++
63[a]	8,16-$C_{35:2}$	3467	++	++	++	++	++	++	++	++	+	+	++	++	++	+++	+++	++	++	++	++	++

Table 1 (continued)

No.	Hydrocarbon	RI[c]	Female									Male								
			day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30	day 0	day 1	day 2	day 3	day 5	day 7	day 14	day 16	day 20	day 30
64[a]	7,18-$C_{35:2}$	3469	++	++	++	++	++	++	++	++	+	+	++	++	++	+++	+++	++	++	++	++	++
65[a]	13-$C_{35:1}$	3480	++	++	++	++	++	++	++	++	+	+	++	++	++	+++	+++	++	++	++	++	++
66[a]	x,y-$C_{37:2}$	3668	++	++	++	++	++	++	+	+	+	-	++	++	++	++	+++	++	++	++	++	++

tr≤0.3 %; +≤1 %; ++<5 %; +++<10 %; ++++>10 %

[a] Compounds included in the statistical analysis (N=5). The CHCs which were present only in trace amounts were excluded from the multivariate analyses (some peaks had to be combined as they were not always clearly separated in the GC profile, and some others could not always be reliably detected and were thus omitted from the analysis).

[b] Male-specific compounds

[c] RI Retention index

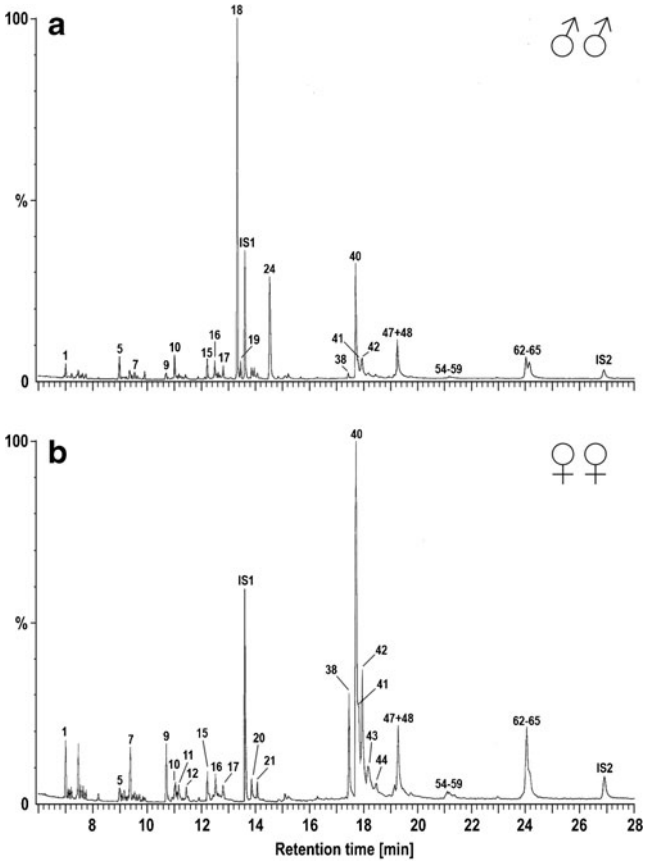

Fig. 1 Cuticular hydrocarbon profiles obtained by gas chromatography (GC-EI/MS) of the hexane extract of twenty-day-old virgin male (**a**) and female (**b**) *Anastrepha fraterculus*. The *numbered peaks* correspond to Table 1. Abbreviations: *IS1* Internal standard 1, *IS2* Internal standard 2

extracted from the 53 CHC compounds analyzed (marked with a, in Table 1, for the loadings of the PCs, see Suppl. Table 1). The compounds which were present in trace amounts were not included in the DA. Some of the co-eluting peaks were combined for the DA. The canonical DA functions separated the male from female CHC pattern (Fig. 3; P<0.001).

Age-Specific Differences in CHC Amount and Composition The absolute amount of CHCs changed with age in males, with a tendency towards lower amounts in older animals, but not in females (Suppl. Fig. 1). Cluster analyses, DA, and MANOVA revealed marked changes of cuticular profiles depending on age in males and females. The discriminant analysis (DA) significantly differentiated the 20 age/gender groups (10 age groups for males and 10 for females) (*Wilk's Lambda*<0.001, X^2=1875.6, df=171, P <0.001), and cross-validation resulted in 97 % correct classifications of the originally grouped cases. Both DA and cluster analyses indicated that the CHC profiles were similar for emerging males and females, and remained similar for

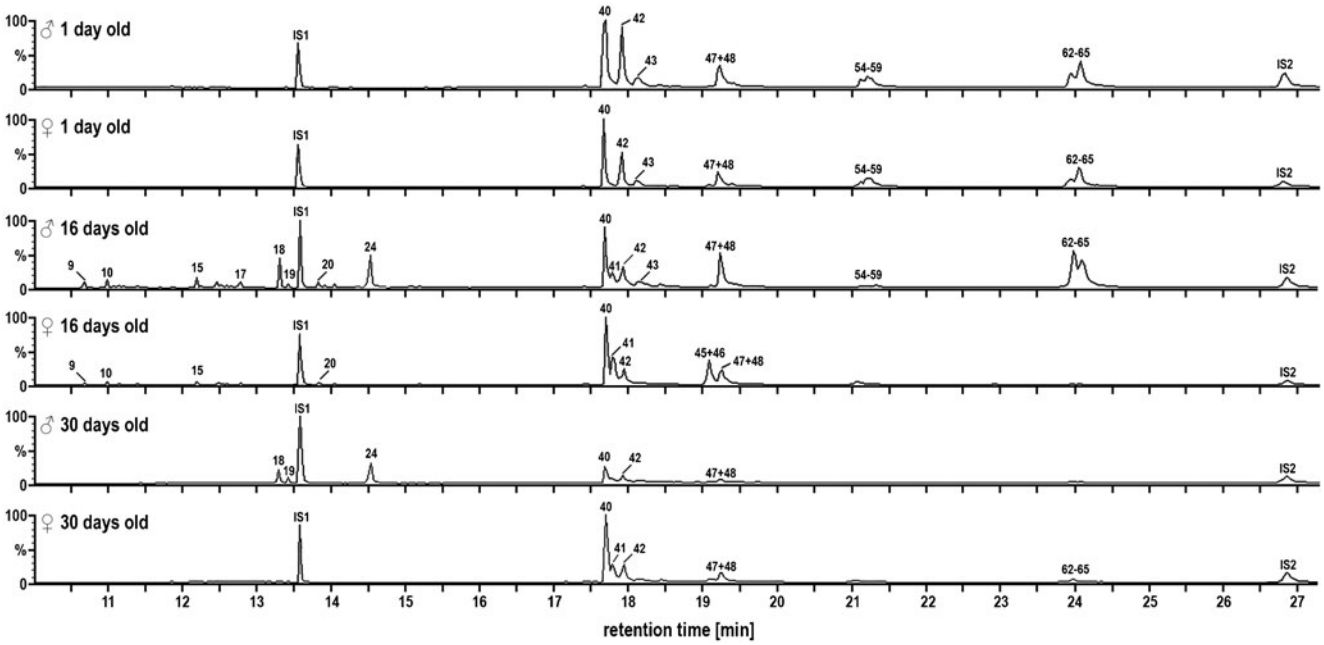

Fig. 2 Total ion current chromatograms of the cuticular crude hexane extracts from male and female *Anastrepha fraterculus* of different ages. The *peak numbers* correspond to those in Table 1. Abbreviations: *IS1* Internal standard 1, *IS2* Internal standard 2

Table 2 Double-bond locations and characteristic ions of the alkenes and alkadienes identified in *Anastrepha fraterculus* by acetonitrile chemical ionization and MS/MS of the $[M+54]^+$ adduct ions

Hydrocarbon	RI[a]	$[M+54]^+$	MS/MS fragments
7-$C_{21:1}$	2083	248	180/278
7-$C_{22:1}$	2182	362	180/292
7-$C_{23:1}$	2283	376	180/306
7-$C_{25:1}$	2483	404	180/334
9-$C_{29:1}$	2883	460	208/362
9,11-$C_{31:2}$	3067	486	222/374
8,14-$C_{31:2}$	3067	486	262/388
11,14-$C_{31:2}$	3067	486	262/346
11-$C_{31:1}$	3081	488	236/362
10-$C_{31:1}$	3081	488	222/376
9,15-$C_{33:2}$	3260	514	276/402
9,16-$C_{33:2}$	3266	514	290/402
9,17-$C_{33:2}$	3268	514	304/402
7,13-$C_{33:2}$	3269	514	248/430
7,19-$C_{33:2}$	3269	514	332/430
11-$C_{33:1}$	3280	516	236/390
7-$C_{33:1}$	3283	516	180/446
9,16-$C_{35:2}$	3466	542	290/430
8,16-$C_{35:2}$	3467	542	290/444
7,18-$C_{35:2}$	3469	542	318/458
13-$C_{35:1}$	3480	544	264/390
x,y-$C_{37:2}$	3668[b]	570	-

[a] RI Retention index

[b] extrapolated (calculated) RI

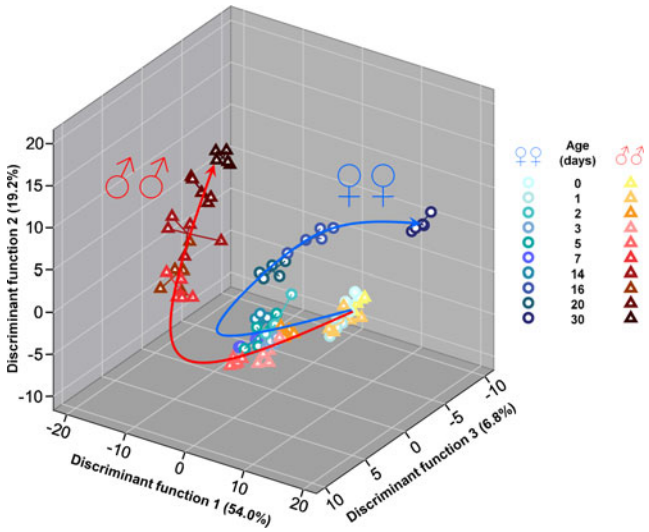

Fig. 3 Discriminant analysis of *Anastrepha fraterculus* male (*red*) and female (*blue*) CHC profiles, based on nine principal components extracted from the 53 CHC compounds analyzed (some peaks had to be combined as they were not always clearly separated in the GC profile, and some others could not always be reliably detected and were thus omitted from the analysis). The first three discriminant functions that cumulatively retained 80.0 % of the variance are given. The *colors* represent ten age groups of both females and males, respectively (days 0 to 30 after eclosion). The lines connect samples to their age-group centroids. The *arrows* indicate the changes in CHC profiles with increasing age for males (*red*) and females (*blue*). Note that the chemical profiles of the males and females are very similar during the first days after eclosion but diverge strongly after 1–2 weeks

both sexes until about days 5–7 after emergence. Then, the CHC profiles of males and females started to diverge and ended up being completely different in the older flies (days 20 and 30) (Fig. 3). The MANOVA for both males and females confirmed that the CHCs profiles change across the 10 age groups (males: *df*=32.0, *Wilk's Lambda*<0.001, *F*= 45.9, *P*<0.001; females: *df*=40.7, *Wilk's Lambda*<0.001, *F*= 56.3, *P*<0.001), and revealed significant differences across age groups for all of the individual compounds (*P*<0.05). In males, the relative proportions of several compounds increased continuously with age (3-MeC$_{12}$, 9-MeC$_{15}$, *n*-C$_{16}$, *n*-C$_{17}$, 7-C$_{21:1}$, 7-C$_{22:1}$, and 7-C$_{23:1}$,) while some others decreased (*n*-C$_{29}$ and 7,19-C$_{33:2}$). Similarly, in females some of the same compounds showed consistent age-related changes increasing with age (3-MeC$_{12}$, 9-MeC$_{15}$, 4-MeC$_{15}$, *n*-C$_{16}$, *n*-C$_{17}$, and *n*-C$_{31}$). In contrast to the males, however, the effects were reversed for most of these compounds in very old (30 days) females (except for *n*-C$_{17}$ and *n*-C$_{31}$).

Discussion

The cuticular hydrocarbons (CHCs) of the males and females of an *A. fraterculus* laboratory population from Argentina were characterized. The CHC profile of this species is a complex mixture of straight-chained and methyl-branched saturated and unsaturated hydrocarbons with a wide range of carbon backbones (C$_{13}$–C$_{37}$). The number and type of CHCs that we identified are in agreement with the previous reported analyses for other tephritid fruit fly species: 39 CHCs in *Anastrepha* larvae (4 alkanes, 23 monomethyl alkanes, 10 dimethyl alkanes, 1 alkene, and 1 unidentified CHC) (Sutton and Carlson, 1993), 11 CHCs in the larvae and adults of *A. ludens* and *A. suspensa* (2 alkanes, 9 monomethyl alkanes), 7 CHCs in the larvae and adults of *Ceratitis capitata* and *C. rosa* (2 alkanes, 5 monomethyl alkanes), 29 CHCs in the larvae and adults of *Dacus cucurbitae* and *D. dorsalis* (3 alkanes, 25 monomethyl alkanes, and 1 unidentified) (Carlson and Yocom, 1986), and 40 peaks in the adults of *Bactrocera malaysia* (12 alkanes, 37 monomethyl alkanes, 15 dimethyl alkanes, 11 unknown compounds) (Goh et al., 1993). The retention indices were used by Lavine et al. (1992) and Sutton and Steck (1994) for the characterization of the CHCs of *Anastrepha* and *Ceratitis* larvae. However, in comparison with previous reports, we identified a higher number of *n*-alkanes, short-chain branched alkanes, long-chain alkenes, and alkadienes.

Sex-Specificity The CHC profiles of mature flies are clearly sex-specific, both in quality and quantity as evidenced by a multivariate analysis of the GC data. The CHCs responsible for the separation between the two sexes were mainly the male alkenes, which have been previously published as major components of the CHC profile in both sexes of *Drosophila simulans* and in the males of *D. melanogaster* (Rouault et al., 2004). Qualitative differences in the CHC profiles of males and females also have been reported for other Tephritidae (Carlson and Yocom, 1986; Goh et al., 1993). The sex-specificity of the CHC profiles may be driven by sexual selection if the chemical composition of the cuticle is used as a pheromonal signal in mate choice, as is the case e.g., in *D. serrata* (Chenoweth and Blows, 2005; Thomas and Simmons, 2008). There is evidence that male-specific cuticular pheromones can influence a female's propensity to mate in *D. melanogaster* (Grillet et al., 2006). Although the precopulatory behavior of *A. fraterculus* has been intensively studied (Morgante et al., 1983; Lima et al., 1994; Lima and Howse, 1997; Aluja and Norrbom, 2001; Segura et al., 2007; Gomez et al., 2011), it is not yet known whether chemical CHC signals are an essential part of the multimodal premating signaling, or which behavioral decisions are based on chemical information. Such knowledge is essential for designing experiments to investigate the role of CHCs in *A. fraterculus* courtship behavior. Nevertheless, the function of CHCs in *Anastrepha* courtship and mating remains unclear and has yet to be investigated.

Age-Specificity Our analyses revealed marked age-related changes in the CHC profile of both males and females. The quantitative age-dependent dynamics in the CHC profiles had not yet been reported in *Anastrepha*. Young flies of both sexes (days 0–5 after eclosion) were similar regarding the CHC composition. After day 7, the profiles clearly diverged and ended up being completely different between older males and females (days 20 and 30). A number of studies have demonstrated that the differences in the cuticular hydrocarbons between sexes can be more marked in older than they are in younger individuals (Brown et al., 1992; Mpuru et al., 2001; Caputo et al., 2005; Thomas and Simmons, 2008). *Anastrepha fraterculus* reaches sexual maturation at the mean age of 10 days after eclosion, when males start to release a long-range sex pheromone that attracts females for mating (Lima et al., 1994, 2001). Our findings about the differentiation of the CHC composition from the 7th day after eclosion correlate with the period of the release of the sex pheromone by males. The age-dependent changes of the CHC profiles in males and females may represent a cue for sex recognition and mate choice (Blomquist and Bagnères, 2010). The differences also could be related to dynamics in hormone levels. Further analyses and bioassays would be necessary to test these hypotheses in order to learn more about sexual selection and mate choice in *A. fraterculus*.

Method Development The combination of the different gas chromatographic-mass spectrometric methods allowed for a

comprehensive characterization of the CHC composition in both males and females of *A. fraterculus*. Each of the analytical methods used (GC-EI/MS, GC×GC/TOFMS, GC-CI/MS/MS) is well suited for cuticular hydrocarbon analyses, but differs considerably in the amount of information provided. GC×GC/TOFMS combines the high separation power of two-dimensional chromatography and mass spectrometry, which allows the acquisition of detailed information about almost all of the components of the CHC mixtures. On the other hand, it is not possible to use this method to identify the position of the double-bond in unsaturated CHCs. It was, therefore, necessary to use GC-CI/MS with acetonitrile that formed an *m/z* 54 radical anion, which reacts with the double bond(s) in CHCs, and the collision-induced-dissociation experiments provide information on the double-bond position. Our detailed analysis and identification of CHCs can be used as a baseline for the further exploration of the cryptic species hidden within the South American fruit fly *Anastrepha fraterculus* complex. For screening purposes, the analysis can be performed on the more widely available GC with a flame-ionization detector, and the RI determined here can be used for CHC identification. In the near future, the mass spectrometric imaging of males and females will be performed using a previously developed protocol (Vrkoslav et al., 2010). The imaging data can show whether some of the CHCs are spatially localized on the flies and may direct male/female sexual contacts.

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Unsaturated Cuticular Hydrocarbons Synergize Responses to Sex Attractant Pheromone in the Yellow Peach Moth, *Conogethes punctiferalis*

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Abstract Four trienyl hydrocarbons, (*Z*3, *Z*6, *Z*9)-tricosatriene (Z3, Z6, Z9-23:HC), (*Z*3, *Z*6, *Z*9)-pentacosatriene (Z3, Z6, Z9-25:HC), (*Z*3, *Z*6, *Z*9)-heptacosatriene (Z3, Z6, Z9-27:HC), and (*Z*3, *Z*6, *Z*9)-nonacosatriene (Z3, Z6, Z9-29:HC) were identified in a non-polar fraction of the body wax of male and female yellow peach moth, *Conogethes punctiferalis*. The relative amounts and ratios of these hydrocarbons differed between sexes. In females, the ratios in body wax and pheromone gland extracts were similar, with lesser amounts found in gland extracts. Synergistic effects of these hydrocarbons when added to the known aldehyde pheromone components were assessed in wind tunnel tests. A blend of (*E*)-10-hexadecenal (E10-16: Ald) and (*Z*)-10-hexadecenal (Z10-16: Ald) elicited upwind flight and orientation of males to the pheromone source, but arriving males did not remain close to source for very long. Among the hydrocarbons identified, only Z3, Z6, Z9-23:HC enhanced the activity of the aldehyde blend by increasing the time spent close to the source and the number of source contacts. Z3, Z6, Z9-23:HC and (*Z*9)-heptacosene (Z9-27:HC) also increased close-range responses to the aldehyde blend. The activity of the aldehyde blend plus these two hydrocarbons was similar to that of crude pheromone extract. Positive dose-response relationships between the aldehyde blend and two hydrocarbon mixtures were found. The lowest doses that elicited synergism were 10^{-1} female equivalents (of body wax extracts) for the two hydrocarbons, and 10^{-2} female equivalents for the total unsaturated hydrocarbon mixture.

Keywords Unsaturated cuticular hydrocarbons · Synergist · Sex pheromone · Lepidoptera · Crambidae · Insect pest · Short-range signal

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Introduction

The yellow peach moth, *Conogethes punctiferalis* (Guenée) (Lepidoptera: Crambidae), is distributed through tropical and eastern Asia and Australia, where it causes damage to many orchard, spice, and vegetable crops, including peach, chestnut, durian, citrus, papaya, cardamom, ginger, eggplant, and maize (Sekiguchi, 1974; Waterhouse, 1993). Konno et al. (1982) identified (*E*)-10-hexadecenal (E10-16:Ald) as a sex pheromone component in pheromone glands of females, and reported a binary mixture (90:10) of E10-16:Ald and (*Z*)-10-hexadecenal (Z10-16:Ald) as an effective pheromone lure in field tests. These two aldehydes also were identified as sex pheromone components, with small alterations in ratios, for Chinese and Korean populations of this species (Liu et al., 1994; Jung et al., 2000). However, pheromone traps baited with this binary aldehyde blend did not perform well in some regions of Japan (Kondo et al., 2008), thus reducing the reliability of this technique

for monitoring this pest. Two additional compounds [*n*-hexadecanal and (*E*)-10-hexadecen-1-ol] that elicited responses from antennae of male moths were identified in Japanese populations of this species by coupled gas chromatography-electroantennogram detection (GC-EAD). However, when added to lures containing E10-16:Ald and Z10-16:Ald, neither of these components improved the attractiveness of lures (Kimura, 2002).

We hypothesized that there might be some unknown components with low volatility that were essential to the pheromone of yellow peach moth. First, we found that non-polar fractions of pheromone gland extracts and body wax extracts of females, mixed with the aldehyde pheromone blend, synergistically enhanced male responses close to pheromone sources (Xiao and Honda, 2010). Then, using wind tunnel bioassay-driven fractionation, we found that 3 % and 50 % ether/hexane fractions of pheromone gland and female body wax extracts, from an $AgNO_3$-impregnated silica gel column, increased attraction to the aldehydes. The components in the 3 % ether fraction were identified as a series of homologous monounsaturated hydrocarbons, among which (*Z*)-9-heptacosene (Z9-27:HC) enhanced male responses at short distances from the aldehyde source. However, blends of Z9-27:HC, or the entire monoene fraction, added to the aldehydes were still not as active as blends of the aldehydes with crude extracts of female body wax (Xiao et al., 2011), suggesting that there were additional active components in the 50 % ether/hexane fraction.

In this paper, we report the identification of several polyunsaturated hydrocarbons in the 50 % ether/hexane fraction from analyses of male and female body wax, and extracts of pheromone glands of females, by liquid chromatography on columns of silica gel impregnated with silver nitrate. We also report the synthesis and testing of these polyunsaturated hydrocarbons and the previously identified monoenes as possible pheromone synergists that enhance the responses of male moths when close to an aldehyde pheromone source.

Methods and Materials

Insects A colony of yellow peach moth was started from larvae collected in chestnut fields at Ibaraki prefecture, Japan. In the laboratory, larvae were reared on an artificial diet and chestnuts or corn (Honda et al., 1979). Pupae were sexed and the sexes kept separately in cages at 25±1 °C, 40–60 % R.H., under a 15 L: 9 D photoperiod. Adults were provided with a 10 % sugar solution on cotton pads. A 15 W red, incandescent lamp was used for observations during the scotophase.

Preparation and Fractionation of Extracts The terminal abdominal segments including the sex pheromone glands were excised with micro-scissors from 2 to 4 d-old virgin female moths anesthetized with CO_2, 5–8 h after lights off. The abdominal tips were extracted for 15 min at room temperature with redistilled *n*-hexane (ca. 1 μl per insect) containing 0.01 % butylated hydroxytoluene (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) as an antioxidant. Crude pheromone extracts were pooled in screwcap vials [200 female equivalents (FE) per vial] with Teflon®-lined caps, and stored at −20 °C until use. Body wax extracts were prepared from 2 to 4 d-old virgin females and males, which were anesthetized during the photophase (4–6 h after lights on) to avoid contamination of extracts from females with the aldehyde pheromone components. A whole insect was dipped in redistilled *n*-hexane for 2 min in a small glass vial. Body wax extracts were passed through a cotton filter in a Pasteur pipette® to remove body scales. Extracts of 200 insects were pooled in each vial, and stored at −20 °C.

Crude pheromone gland extracts and body wax extracts of females and males were concentrated under a N_2 stream and subjected to liquid chromatography (1 cm diam.×3 cm long) on 1 g of Florisil (60–100 μm particle size, Wako Pure Chemicals) impregnated with 7 % distilled water (Carroll, 1961). Extracts were successively eluted with 10 ml each of hexane and 5 % ether in hexane. The first eluate of each extract contained mainly hydrocarbons (non-polar fraction, NPF), whereas the second, slightly more polar, fraction of crude pheromone gland extracts contained E10-16:Ald and Z10-16:Ald. The NPF was further fractionated on a column, packed with 1 g of C-200 silica gel (1 cm diam.×3 cm long, particle size: 75–150 μm, Wako Pure Chemicals) impregnated with 10 % $AgNO_3$. The column was successively eluted with 10 ml each of hexane, 1, 3, 10, 30, and 50 % ether in hexane, and finally with 10 ml ether. Fractions were concentrated under N_2 to 1 FE (female equivalent)/μl or 1 male equivalent/μl, and stored at −20 °C until use.

Analyses of Extracts Gas chromatography-mass spectrometry (GC-MS) analyses were conducted with a Hewlett-Packard 5972 mass selective detector coupled to an HP 5890 SeriesIIGC, equipped with an HP-5ms column (30 m×0.25 mm ID, film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA). The oven temperature was held at 140 °C for 2 min, then increased to 280 °C at 5 °C.min^{-1}, and held at 280 °C for 10 min. Samples were injected in splitless mode at 280 °C, with He as carrier gas. The MS interface was maintained at 280 °C.

Chemicals E10-16:Ald and Z10-16:Ald were obtained from Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan), and a mixture of these two aldehydes (ratio of 95.5:4.5) was prepared after

purification (each 99.5 % pure) by chromatography over 10 % $AgNO_3$-impregnated silica gel. (*Z*)-9-Tricosene (Z9-23:HC), (*Z*)-9-nonacosene (Z9-29:HC), (*Z*)-9-pentacosene (Z9-25:HC), Z9-27:HC, and (*Z*)-9-hentricontene (Z9-31:HC) were synthesized by *Z*-selective Wittig reactions of the corresponding aldehydes with the C9-ylide generated by mixing KHMDS and *n*-nonyltriphenylphosphonium bromide, as described in Xiao et al. (2011). Four trienyl hydrocarbons were synthesized by reduction of the linolenic ester to the corresponding alcohol, followed by tosylation and chain extension with the appropriate dialkyllithium cuprates or alkylmagnesium bromides (Conner et al., 1980; Underhill et al., 1983). The synthetic triene hydrocarbons were purified on a 20 % $AgNO_3$–impregnated silica gel column. Purities of the trienes were confirmed to be more than 99.0 % by GC-MS. (*Z*3, *Z*6, Z9)-Heptacosatriene was used as an external standard for quantification of polyene hydrocarbons in body wax and pheromone gland extracts.

Bioassays All bioassays were conducted in an acrylic wind tunnel (2 m long×0.3 m diam.) as described in Xiao and Honda (2010). All wind tunnel tests were conducted at 25±1 °C and 40–60 % RH under a red incandescent light at ca. 2 lux. One μl of a test solution in *n*-hexane was loaded on a triangular filter paper (0.5 cm base×1.5 cm height), and the filter paper hung 15 cm above the floor and 15 cm from the upwind end of the wind tunnel. Two to 4-d-old males were acclimatized first in a wooden cage (20×20×30 cm) for 24 h and then individually in a small metal mesh cage (6 cm diam×6 cm high) for 1 h. A cage containing a male moth was hung 15 cm below the ceiling at the downwind end of the wind tunnel. Male moths were allowed to leave the cage just after setting a stimulus source at the upwind end of the tunnel, and the following four behaviors recorded for 3 min: (1) flight initiation; (2) orientation to the plume (male moth flew upwind along the plume); (3) remained close to source (hovered within 10 cm of the source); and (4) source contact (Mazor and Dunkelblum, 1992; Xiao and Honda, 2010). The time spent close to the source and the numbers of source contacts were also recorded.

Statistical Analysis Data on initiating flight, orientating to the plume, and remaining close to the source were first analyzed by n×2 Fisher's exact probability test using the actual number of insects. When probability was significant ($P<0.05$), multiple comparisons were performed employing Ryan's method. Data on mean time for remaining close to the source and mean numbers of source contacts by male moths were analyzed by Tukey's test. Software package R, version 2.10.0 (R Development Core Team, 2009), was used for the statistical analyses.

Results

Identification Four major peaks (a: 19.9 min; b: 23.1 min; c: 26.2 min; d: 29.0 min; area percent of total peak area: 93 %) were detected by GC analysis of the fraction of female body wax extracts that eluted with 50 % ether/hexane from an $AgNO_3$-impregnated silica gel column (Fig. 1). The mass spectra of these compounds were similar. In each spectrum, molecular ions were observed at *m/z* 318 ($C_{23}H_{44}$), 346 ($C_{25}H_{48}$), 374 ($C_{27}H_{52}$), and 402 ($C_{29}H_{56}$), respectively. All spectra contained ions at *m/z* 79 and *m/z* 108, which were assigned as $[(CH{=}CH)_3H]^+$ and $[CH_3CH_2\ (CH{=}CH)_3H]^+$, respectively, and which are diagnostic for methylene-interrupted 3,6,9-alkatriene structures (Millar, 2000). Distinct fragment ions also were observed at *m/z* 262, 290, 318, and 346 in each spectrum, respectively, corresponding to M^+-56 (loss of C_4H_8); i.e., $[H(CH{=}CH)_3C_{13}H_{27}]^+$, $[H(CH{=}CH)_3C_{15}H_{31}]^+$, $[H(CH{=}CH)_3C_{17}H_{35}]^+$, and $[H(CH{=}CH)_3C_{19}H_{39}]^+$. On the basis of their mass spectra, these four compounds were tentatively identified as 3, 6, 9-tricosatriene, 3, 6, 9-pentacosatriene, 3, 6, 9-heptacosatriene, and 3, 6, 9-nonacosatriene, respectively. The shorter retention times of these compounds, compared to those of corresponding alkanes, on a non-polar column verified that none of the double bonds in these molecules were conjugated (Millar, 2000). The mass spectra and retention times of the four compounds matched those of synthetic Z3, Z6, Z9-23:HC, Z3, Z6, Z9-25:HC, Z3, Z6, Z9-27:HC, and Z3, Z6, Z9-29:HC (peaks a–d, respectively).

The same trienyl hydrocarbons also were identified in the 50 % ether/hexane fraction of extracts of pheromone glands of females, and in body wax extracts of male moths. The quantitative profiles of the hydrocarbons from the female body wax extracts, the pheromone gland extracts, and male body wax extracts are shown in Table 1. Ratios (as peak area percentage) of the four hydrocarbons were similar between the female body wax and pheromone gland extracts, but markedly different from male body wax extracts, which contained relatively little Z3, Z6, Z9-23:HC in comparison to the extracts from females. The total amount of triene hydrocarbons from the female body wax extracts was ca. 9.4 ng/female, about 10 times more than that in pheromone gland extracts, but 37 times less than in male body wax extracts.

Synergistic Effects of Unsaturated Hydrocarbons We had previously shown that the NPF of female body wax was not by itself attractive to male moths (Xiao and Honda, 2010). The results of bioassays testing for synergism between the four trienyl hydrocarbons found in the NPF of female body wax and the aldehyde sex pheromone blend are summarized in Table 2. As reported previously, the aldehyde blend alone elicited orientation of males to the pheromone source, but

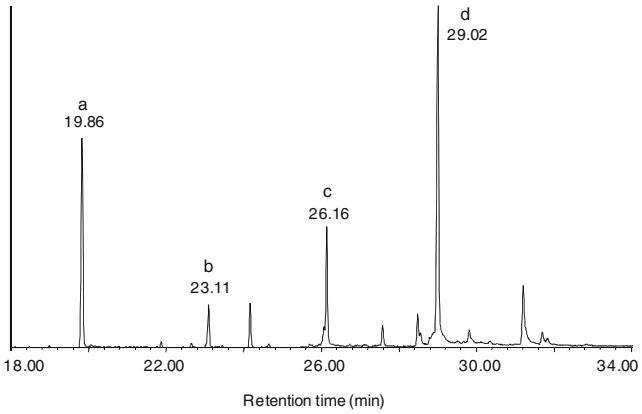

Fig. 1 Total ion chromatogram of the 50 % ether/hexane fraction of the non-polar fraction of female *Conogethes punctiferalis* body wax extract separated on a $AgNO_3$-impregnated silica gel column. Four major peaks (**a**–**d**) account for 93 % of total peak area. Compound identities: **a**=(*Z*3, *Z*6, *Z*9)-tricosatriene, **b**=(*Z*3, *Z*6, *Z*9)-pentacosatriene, **c**=(*Z*3, *Z*6, *Z*9)-heptacosatriene, and **d**=(*Z*3, *Z*6, *Z*9)-nonacosatriene. Conditions: HP-5ms column (30 m×0.25 mm ID, 0.25 μm film; 140 °C for 2 min, then to 280 °C at 5 °C.min^{-1}, 280 °C for 10 min). Samples were injected in splitlessly mode

once attracted, males did not remain close to the source. When each of the four trienes were tested as mixtures with the aldehyde blend, the orientation behavior of males did not change. However, the number of source contacts was enhanced by the addition of Z3, Z6, Z9-23:HC. The mixture of all the trienes and the aldehyde blend also induced males to spend more time close to the source, and increased the number of source contacts in comparison to the aldehyde blend alone.

Although Z3, Z6, Z9-23:HC alone or the mixture of all four trienes enhanced responses to the aldehyde blend, the responses were not as high as those elicited by the non-polar fraction of female body wax extracts or crude pheromone extracts. Similar reduced responses, compared to whole extracts, had also been observed by addition of Z9-27:HC and a mixture of other monoenes (Xiao et al., 2011). Thus, we tested the effects of mixtures of the monoenes and trienes added to the aldehyde blend. As shown in Table 3, similar response rates were found among all treatments for flight initiation, orientation to the plume, and arrestment close to the source. In comparison to the aldehyde blend alone, males remained close to the source longer, and the mean number of source contacts was higher, to the combination of Z9-27:HC, Z3, Z6, Z9-23:HC and the aldehyde blend. This activity was equivalent to that of crude pheromone extracts, and to the combinations of all monoenes and Z3, Z6, Z9-23:HC with the aldehyde blend, and all monoenes and all trienes with the aldehyde blend. In the remaining treatments, i.e., the aldehydes plus all trienes without Z3, Z6, Z9-23: HC, and the aldehydes with all monoenes and trienes except Z9-27: HC and Z3, Z6, Z9-23: HC, no enhanced effect, compared to responses to the aldehyde blend alone, was found.

The dose-response relationships for a mixture of Z9-27: HC, Z3, Z6, Z9-23:HC, and aldehydes, and for a mixture of all monoenes and trienes and aldehydes, were tested. Similar patterns between dose and mean time remaining close to the source, and dose and mean number of source contacts were observed, so only the relationship between dose and mean time remaining close to source is shown in Fig. 2. Thus, for the blend of Z9-27:HC, Z3, Z6, Z9-23:HC, and the aldehydes, the lowest dose that elicited an enhanced effect was 10^{-1} FE (of body wax extracts), whereas for the mixture of all monoenes, trienes, and aldehydes it was 10^{-2} FE. No difference between responses to the two mixtures was found for doses of 10^{-1} FE or 1 FE.

Discussion

In order to improve the relatively poor performance of the pheromone lure for the yellow peach moth, we searched for

Table 1 Ratios and amounts of triene hydrocarbons found in the Female Body Wax (FBW), Pheromone Gland (PG) and Male Body Wax (MBW) extracts of *Conogethes punctiferalis*

Trienes[a]	Ratio (%)[b, c]			Amount (ng/ insect) [c]		
	FBW	PG	MBW	FBW	PG	MBW
Z3, Z6, Z9-23:HC	25.2±0.4	33.7±1.0	0.1±0.0	2.4±0.1	0.3±0.0	1.0±0.0
Z3, Z6, Z9-25:HC	7.4±0.4	5.6±0.8	4.6±0.1	1.4±0.1	0.1±0.0	18.6±0.7
Z3, Z6, Z9-27:HC	27.0±1.0	15.2±1.7	41.4±0.4	2.5±0.1	0.2±0.0	141.2±7.9
Z3, Z6, Z9-29:HC	40.5±1.0	45.4±2.7	53.9±0.3	3.2±0.2	0.3±0.0	182.7±8.3
Total	–	–	–	9.4±0.4	0.9±0.1	343.6±16.7

[a] Trienes: (*Z*3, *Z*6, *Z*9)-tricosatriene (Z3, Z6, Z9-23:HC), (*Z*3, *Z*6, *Z*9)-pentacosatriene (Z3, Z6, Z9-25:HC), (*Z*3, *Z*6, *Z*9)-heptacosatriene (Z3, Z6, Z9-27:HC) and (*Z*3, *Z*6, *Z*9)-nonacosatriene (Z3, Z6, Z9-29:HC)

[b] Ratios are shown as the integrated peak area of each triene as a percentage of the total area of all triene hydrocarbons

[c] Values are shown as the mean±standard deviation, from 3 replicates

Table 2 Behavioral responses of *Conogethes punctiferalis* males to mixtures of triene hydrocarbons plus the aldehyde blend in wind tunnel tests

Treatment [a, b] (+Aldehyde blend)	N	Male orientation (%) [d]			Male remaining close to source [e]	
		Initiate flight	Orientate to plume	Close to source	Mean time (sec)	Mean number of source contacts
Z3, Z6, Z9-23:HC	19	100 a	94.7 a	89.5 a	30.6±2.8 ab	11.2±1.1 a
Z3, Z6, Z9-25:HC	17	100 a	94.1 a	94.1 a	16.2±2.5 c	5.3±1.0 b
Z3, Z6, Z9-27:HC	18	100 a	88.9 a	88.9 a	17.5±2.0 c	5.2±0.8 b
Z3, Z6, Z9-29:HC	17	100 a	94.1 a	94.1 a	17.6±3.0 c	4.8±1.0 b
Mixture [c]	20	100 a	90.0 a	90.0 a	34.3±4.8 a	12.5±1.7 a
Control	24	100 a	95.8 a	95.8 a	21.1±2.2 bc	6.8±0.9 b

[a] Z3, Z6, Z9-23:HC, Z3, Z6, Z9-25:HC, Z3, Z6, Z9-27:HC and Z3, Z6, Z9-29:HC are abbreviations for (*Z*3, *Z*6, *Z*9)-tricosatriene, (*Z*3, *Z*6, *Z*9)-pentacosatriene, (*Z*3, *Z*6, *Z*9)-heptacosatriene and (*Z*3, *Z*6, *Z*9)-nonacosatriene, respectively

[b] Ten ng (1 female equivalent) of a blend of (*E*)-10-hexadecenal and (*Z*)-10-hexadecenal at a ratio of 95.5:4.5 were added to 10 ng each of all treatments; the control was the aldehyde blend alone

[c] A mixture of all trienes at the natural ratio found in female body wax extracts

[d] Data with different letters in the same column are different, $P<0.05$, by Ryan's multiple comparisons after Fisher's exact probability test

[e] Data with different letters in the same column are different, $P<0.05$, by Tukey's multiple comparisons after ANOVA

new pheromone components, and showed, in wind tunnel tests, that two fractions of crude pheromone gland extract or female body wax extract enhanced short-range male responses to the standard lure of E10-16:Ald and Z10-16:Ald. We initially identified Z9-27:HC as a synergist of the aldehyde blend (Xiao et al., 2011), but bioassays demonstrated that there must be additional compounds. In the work described here, we identified a second synergist, Z3, Z6, 9Z-23:HC. This compound and several homologs were identified as methylene-interrupted trienes. In Lepidoptera, these and related polyene hydrocarbons are probably biosynthesized in oenocyte cells from linoleic or linolenic acid precursors, leading to the production of a series of homologous polyenes with all-*Z* configurations, and with double bonds at the 3, 6, and 9 positions (Schal et al., 1998; Millar, 2000). Our conclusion is also supported by the fact that all of the 3, 6, 9-polyene hydrocarbon pheromones found to date in pyralid and crambid species have *Z* configurations

Table 3 Behavioral responses of *Conogethes punctiferalis* males to mixtures of the aldehyde blend, monoene and triene hydrocarbons identified in female body wax extracts

Treatment [a, b] (+Aldehyde blend)	N	Male orientation (%) [e]			Male remaining close to source [e]	
		Initiate flight	Orientate to plume	Close to source	Mean time (sec)	Mean number of source contacts
Z9-27:HC+Z3,Z6,Z9-23:HC	20	100 a	95.0 a	95.0 a	94.1±8.5 a	40.8±3.9 a
Monoenes[c]+Z3,Z6,Z9-23:HC	17	100 a	88.2 a	88.2 a	97.0±7.3 a	39.6±3.4 a
Monoenes[c]+Trienes[d]	20	100 a	95.0 a	95.0 a	90.6±7.6 a	38.1±3.2 a
Crude pheromone extracts	14	100 a	87.3 a	87.3 a	88.1±6.7 a	39.7±7.3 a
Trienes[d]-Z3,Z6,Z9-23:HC	16	100 a	93.8 a	93.8 a	19.7±2.8 b	6.3±1.5 b
Monoenes[c]+Trienes[d]-Z9-27:HC-Z3,Z6,Z9-23:HC	19	100 a	94.7 a	94.7 a	35.6±4.0 b	12.2±1.6 b
Control	19	100 a	94.7 a	94.7 a	23.4±3.0 b	7.2±0.9 b

[a] Ten ng (1 female equivalent, FE) of a blend of (*E*)-10-hexadecenal and (*Z*)-10-hexadecenal at a ratio of 95.5:4.5 were added to all treatments (at 3 FE), except that of the crude pheromone extract; the control was the aldehydes blend alone

[b] Z9-27:HC and Z3,Z6,Z9-23:HC are abbreviations for (*Z*9)-heptacosene and (*Z*3, *Z*6, *Z*9)-tricosatriene

[c] Three FE of a monoenes mixture, including (*Z*)-9-tricosene, (*Z*)-9-pentacosene, (*Z*)-9-heptacosene, (*Z*)-9-nonacosene and (*Z*)-9-hentricontene, at the natural ratio in female body wax extracts

[d] Three FE of a trienes mixture, including (*Z*3, *Z*6, *Z*9)-tricosatriene, (*Z*3, *Z*6, *Z*9)-pentacosatriene, (*Z*3, *Z*6, *Z*9)-heptacosatriene and (*Z*3, *Z*6, *Z*9)-nonacosatriene, at the natural ratio in female body wax extracts

[e] Data with different letters in the same column are different, $P<0.05$, by Ryan's multiple comparisons after Fisher's exact probability test

[f] Data with different letters in the same column are different, $P<0.05$, by Tukey's multiple comparisons after ANOVA

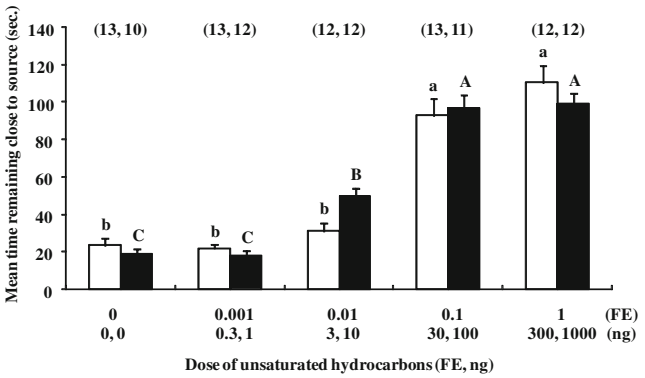

Fig. 2 Dose-response relationships of the mean time remaining close to source by males in wind tunnel tests when (*Z*9)-heptacosene and (*Z*3, *Z*6, *Z*9)-tricosatriene (*open columns*), or the blend of all monoenes and trienes (*closed columns*), were added to the aldehyde blend (10 ng). Values are shown as mean+standard error. *Numbers in parentheses* are the replications of different individual insects. Amounts of hydrocarbons were calculated based on female equivalents (FE) of body wax extracts. Values with the same letter were not different, $P < 0.05$, by Tukey's multiple comparisons after ANOVA

(Cabrera et al., 2001; Millar et al., 2005; Gibb et al., 2007; Strong et al., 2008).

The same trienyl hydrocarbons also were found in male body wax extracts, but in different ratios and, with the exception of Z3, Z6, Z9-23:HC, in much larger amounts (Table 3). Qualitative or quantitative sexual dimorphism of cuticular hydrocarbons occurs widely in insects that use cuticular hydrocarbon components as recognition signals between individuals (Howard and Blomquist, 2005). Sexual dimorphism of cuticular hydrocarbons might result from genetic factors (Liimatainen and Jalon, 2007), dopamine regulation of hydrocarbon biosynthesis (Marican et al., 2004), or even differential transport of hydrocarbons by lipophorin proteins. In yellow peach moth, the factors underlying the quantitative dimorphism of trienes between males and females have not been investigated.

We had previously reported that body wax extracts of female and male yellow peach moth contained the same monoene hydrocarbons (Xiao et al., 2011). Our results indicate that there is little sex specificity for most of the unsaturated hydrocarbons in body wax, but that one or more key components may be increased in females to provide a sex-specific signal. In Lepidoptera, components of female sex pheromones generally are produced in specific ratios, and even small changes in ratios may decrease or eliminate the attraction of conspecific males (Cardé and Haynes, 2004). Thus, it is entirely plausible for a component found in both sexes to be a key component of the overall sex-specific signal produced by females. In yellow peach moth, similar amounts of Z3, Z6, Z9-23:HC were found in body wax of both sexes. However, because males do not produce the aldehyde pheromone components, its presence in males is unlikely to elicit responses from other males. In addition, the amount of the trienyl hydrocarbons was much less than that of the monoene hydrocarbons identified previously (Xiao et al., 2011), although even these low amounts synergized the response to the aldehyde blend. Our dose-response experiment suggests that synergism by these compounds is not particularly sensitive to dose, as long as the amount is above a certain threshold.

Although Z3, Z6, Z9-23:HC synergized the aldehyde blend, its effect, or indeed the effect of the mixture of trienes, was not as high as that of the non-polar fraction of body wax extracts. The synergism of the aldehyde blend by Z9-27:HC also was lower than that of the whole non-polar fraction from female body wax extracts (Xiao et al., 2011). However, the combination of Z3, Z6, Z9-23:HC, Z9-27:HC plus the aldehyde blend was as active as crude pheromone extract (Table 3). These results indicate that the complete sex pheromone system of the yellow peach moth is composed of the two aldehyde components, E10-16:Ald and Z10-16:Ald, and at least two unsaturated hydrocarbons, Z9-27:HC and Z3, Z6, Z9-23:HC.

Lepidopteran pheromone components are classified as Type I, which includes C_{10}–C_{18} alcohols, acetates, and aldehydes, Type II, consisting of C_{17}–C_{23} polyunsaturated hydrocarbons or their epoxide derivatives, and a number of miscellaneous components, which are not readily classified (Millar, 2000; Ando et al., 2004). Recently, more complex systems, consisting of blends of both Type I and Type II compounds have been found as sex pheromones, or sex attractants, in some pyralid (Millar et al., 2005; Strong et al., 2008) and crambid species (Cabrera et al., 2001; Gibb et al., 2007). The yellow peach moth is a further example of a crambid species that uses both Type I (E10-16:Ald and Z10-16:Ald) and Type II compounds (Z9-27:HC and Z3, Z6, Z9-23:HC) in its pheromone. Such combinations may be a widespread motif in lepidopteran pheromone chemistry (Millar et al., 2005), and may indicate a shift in the range of compounds that constitute sex pheromone blends in Lepidoptera. Moreover, in the yellow peach moth, Z9-27:HC and Z3, Z6, Z9-23:HC were in both pheromone gland extracts and body wax extracts, indicating that the alkene components are distributed over the body surface (Table 1). In Lepidoptera, Type II pheromone components are synthesized in epidermal cells and transported to the pheromone gland (Schal et al., 1998; Wei et al., 2004; Matsuoka et al., 2006; Ando et al., 2008), but there has been no report of direct evidence for specific sequestration of these hydrocarbons into pheromone glands. Because lepidopteran sex pheromone glands often occur as modified segmental membranes, between the 8th and 9th abdominal segments (Percy-Cunningham and MacDonald, 1987), pheromone gland extracts contain chemicals not only from the sex pheromone gland itself but also from associated tissues in these segments. In the yellow peach moth, the relatively small

amounts of Z9-27:HC and Z3, Z6, Z9-23:HC in crude pheromone extracts may be from contamination from the cuticular hydrocarbons of the 8th and 9th abdominal segments.

Cuticular hydrocarbons are well known as chemical signals in insects, having roles in species, gender, nest mate, and caste recognition, among other functions (Howard, 1993; Howard and Blomquist, 2005). In Lepidoptera, potential semiochemical functions for cuticular chemicals have long been known (Ono, 1977; Rutowski, 1978; Shimizu and Tamaki, 1980). However, such cuticular hydrocarbons only appear to function as signals when presented in combination with more volatile sex attractant pheromone. For example, in the presence of the volatile sex pheromone, saturated cuticular hydrocarbons induced copulation attempts by male *Orgyia leucostigma* (Grant et al., 1987), or induced persistent approaches and contacts to pheromone sources by male *Anarsia lineatella* (Schlamp et al., 2005). In the yellow peach moth, Z9-27:HC and Z3, Z6, Z9-23:HC were not attractive to males (Xiao and Honda, 2010), but enhanced the activity of E10-16:Ald and Z10-16:Ald. This result confirms the essential role of the E10-16:Ald and Z10-16:Ald in elucidating the signal functions of Z9-27:HC and Z3, Z6, Z9-23:HC in the body wax of the yellow peach moth.

Although possible semiochemical functions of cuticular chemicals of lepidopteran species have been postulated for some time (Ono, 1977; Rutowski, 1978; Shimizu and Tamaki, 1980), the behavioral activities elicited by these compounds have generally been carried out in small spaces, such as a cage (Grant et al., 1987; Schlamp et al., 2005). Our wind-tunnel assays, with free-flying moths, provide more robust data on the synergistic functions of cuticular hydrocarbons as critical components of sex attractant pheromones.

Finally, the finding that Z9-27:HC (Xiao et al., 2011) and Z3, Z6, Z9-23:HC are close-range synergistic components of mate attraction is of fundamental importance to improving our understanding of lepidopteran pheromone systems. As described above, it is clear that, for some species at least, attraction to a long-range, volatile pheromone may be mediated over shorter distances by less volatile hydrocarbons associated with the cuticle. More careful observations of the responses of male moths of other species when close to a volatile pheromone source may reveal further examples of short-range signal components. In yellow peach moth, the poor performance of aldehyde-only pheromone lures was probably due to the absence of Z9-27:HC and Z3, Z6, Z9-23: HC, which enhance the activity of the aldehyde pheromone at shorter distances from the source. The addition of Z9-27:HC and Z3, Z6, Z9-23:HC to the two-component aldehyde blend should induce male moths to stay near or within pheromone traps for a longer time, consequently increasing the likelihood of males being caught.

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2,3-Hexanediols as Sex Attractants and a Female-produced Sex Pheromone for Cerambycid Beetles in the Prionine Genus *Tragosoma*

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Abstract Recent work suggests that closely related cerambycid species often share pheromone components, or even produce pheromone blends of identical composition. However, little is known of the pheromones of species in the subfamily Prioninae. During field bioassays in California, males of three species in the prionine genus *Tragosoma* were attracted to 2,3-hexanediols, common components of male-produced aggregation pheromones of beetles in the subfamily Cerambycinae. We report here that the female-produced sex pheromone of *Tragosoma depsarium* "sp. nov. Laplante" is (2*R*,3*R*)-2,3-hexanediol, and provide evidence from field bioassays and electroantennography that the female-produced pheromone of both *Tragosoma pilosicorne* Casey and *T. depsarium* "*harrisi*" LeConte may be (2*S*,3*R*)-2,3-hexanediol. Sexual dimorphism in the sculpting of the prothorax suggests that the pheromone glands are located in the prothorax of females. This is the second sex attractant pheromone structure identified from the subfamily Prioninae, and our results provide further evidence

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of pheromonal parsimony within the Cerambycidae, in this case extending across both subfamily and gender lines.

Keywords 2,3-hexanediol · Mating behavior · Prioninae · Cerambycidae · Longhorned beetle · Pheromone

Introduction

A rapidly growing body of literature indicates that species in the beetle family Cerambycidae exhibit considerable parsimony in pheromone structures, with closely related species often sharing pheromone components, or even apparently using pheromones of identical composition. For example, males of many species in the subfamily Cerambycinae produce pheromones composed of isomers of 3-hydroxy-2-hexanone and/or 2,3-hexanediol, to which both sexes are attracted (e.g., Lacey et al., 2004, 2007, 2008, 2009; Hanks et al., 2007; Ray et al., 2009). Similarly, the terpenoid alcohol (*E*)-6,10-dimethyl-5,9-undecadien-2-ol, termed "fuscumol", is a male-produced pheromone of some species in the subfamily Spondylidinae (Silk et al., 2007), and a species in the subfamily Lamiinae (Fonseca et al., 2010). Fuscumol and its acetate also attract both sexes of many other lamiine species (e.g., Mitchell et al., 2011), suggesting that these compounds may be pheromone components for these species as well. A single pheromone structure also is shared among species of the lamiine genus *Monochamus*, with males of at least five species producing 2-undecyloxy-1-ethanol (Pajares et al., 2010; Teale et al., 2011, Fierke et al. 2012; Allison et al. unpublished).

We recently identified the first sex attractant pheromone, (3*R*,5*S*)-3,5-dimethyldodecanoic acid, secreted in nanogram quantities by a gland on the ovipositor, from a cerambycid species in the subfamily Prioninae (Tribe Prionini), *Prionus californicus* Motschulsky (Rodstein et al., 2009, 2011). Males of at least seven other North American and European *Prionus* species are attracted by a blend of the four stereoisomers of 3,5-dimethyldodecanoic acid, suggesting that (3*R*,5*S*)-3,5-dimethyldodecanoic acid, or one of its isomers, are likely pheromone components for those species as well (Barbour et al., 2011). These findings further suggest that 3,5-dimethyldodecanoic acid represents another pheromone motif that is shared by multiple species in the family Cerambycidae.

In field bioassays, screening a series of known cerambycid pheromones, males of three prionine species in the genus *Tragosoma* (Tribe Meroscelisini) were attracted to 2,3-hexanediols, the same compounds that are male-produced aggregation pheromones of many cerambycine species (Fettköther et al., 1995; Lacey et al., 2004, 2008, 2009; Ray et al., 2009). At the time of writing, the genus is under revision by S. Laplante (Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Ontario, Canada). Here, we describe the identification of a female-produced sex attractant pheromone for *Tragosoma depsarium* "sp. nov. Laplante", and provide evidence from field bioassays and electroantennography that suggests that the congeners *T. pilosicorne* Casey and *T. depsarium* "*harrisi*" LeConte may also use a 2,3-hexanediol isomer as a sex pheromone.

Methods and Materials

Pheromone Chemicals 2,3-Hexanediol, as a mixture of all 4 stereoisomers, was prepared by $LiAlH_4$ reduction of 2,3-hexanedione (Aldrich Chemical, Milwaukee, WI, USA), as described in Hanks et al. (2007). Diastereomerically pure, but racemic, (2*R**,3*R**)- and (2*R**,3*S**)-2,3-hexanediols were made by OsO_4-catalyzed dihydroxylation of (*Z*)- and (*E*)-2-hexenes (GFS Chemicals, Powell, OH, USA), respectively, as described by Lacey et al. (2004), whereas racemic 3-hydroxy-2-hexanone was made as described in Imrei et al. (2012). The four individual 2,3-hexanediol stereoisomers were synthesized by $NaBH_4$ reduction of (*R*)- or (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanones, followed by acid-catalyzed hydrolysis of the THP-protecting groups and chromatographic separation of the resulting pairs of diol diastereomers (Lacey et al., 2008). The resulting diols had stereoisomeric purities as follows: (2*R*,3*R*), >98 % de; (2*R*,3*S*), >97 % de; (2*S*,3*S*), >98 % de; (2*S*,3*R*), >96 % de.

Field Bioassays Field bioassays were conducted in California and Idaho during July and August of 2005, 2009, 2010, and 2011 (maximum daily temperatures 27–33 °C, at most a trace of precipitation). Black flight-intercept panel traps (see Hanks et al., 2007) were used, suspended from tree branches or from frames of plastic irrigation pipe. Traps were positioned 5–10 m apart in linear transects and checked every 2–9 d, at which time treatments were rotated down transects, to control for location effects, and lures replaced as needed. Trap lures were suspended in the center of traps, but the design of lures changed over seasons as more effective lures were developed (see below).

Tragosoma pilosicorne males were first caught during a field-screening bioassay, conducted 25 July to 10 August 2005 at the University of California's James San Jacinto Mountain Reserve (Riverside Co., CA, USA; Table 1), testing attraction to racemic six-, eight-, and ten-carbon 2,3-alkanediols, 2-hydroxy-3-alkanones, and 3-hydroxy-2-alkanones. Lures consisted of cotton dental rolls loaded with 80 mg of test compounds in 1 ml of hexane in uncapped 3.7 ml glass vials, with controls consisting of hexane only.

Attraction of *T. pilosicorne* to (2*R**,3*S**)-2,3-hexanediol was confirmed with a follow-up study conducted at the same site from 10–15 August 2005. Traps were modified so as to capture beetles alive for pheromone analysis

Table 1 Study sites where field bioassays were conducted. All field sites were mixed conifer-oak woodlands

Site name (**abbreviation**)	Location	Position of first trap in transect
Univ. California James San Jacinto Mountain Reserve (**JMR**)	San Jacinto Mountains, Riverside Co., CA	N 33° 48′ 30.00″, W 116° 46′ 40.02″; 1,638 m elevation
Barton Flats (**BF**)	West Jenks Lake Road, San Bernardino National Forest, Mountaintop Ranger District, San Bernardino Co., CA	N 34° 9′ 47″ W116° 53′ 57″; 2,020 m elevation
Blue Ridge (**BR**)	Blue Ridge Truck Trail, Angeles National Forest, Mojave River Ranger District, Los Angeles Co., CA	N 34°22′12″, W 117°42′16.82″, 2,200 m elevation
Rancho los Mochos Boy Scout Camp (**RLM**)	Mines Rd., Foothills of Diablo Mountain Range, Alameda Co., CA	Replicate 1: N37° 32′35″ W121° 34′19″ 763 m elevation; replicate 2: N37° 32′38″ W121° 34′22″ 742 m elevation
University of California Lick Observatory (**UCLO1**)	Old Spring Rd. Mt. Hamilton, Diablo Mountain Range, Santa Clara Co., CA	N37° 20′23″ W121° 38′08″ 1,166 m elevation
University of California Lick Observatory (**UCLO2**)	Mule Trail Rd., Mt. Hamilton, Diablo Mountain Range, Santa Clara Co., CA	N37° 20′58″ W121° 37′12″ 1,155 m elevation
Lone Pine Canyon (**LP**)	Lone Pine Canyon Rd., San Gabriel Mountains, San Bernardino Co., CA	N34° 20′19. 7″ W117° 36′06.4″ 1,825 m elevation
Shafer Butte (**SB**)	National Forest Service Rd. 374, Boise Co., ID	N43° 47′0.28″ W116° 5′12.58″ 2,041 m elevation
Sage Hen Reservoir (**SR**)	National Forest Service Rd. 614, Gem Co., ID	N44° 20′12.17″ W116° 10′43.42″ 2,041 1,552 m elevation

(erroneously assuming that both sexes would be attracted; see Results and Hanks et al., 2007 for details about modification of traps). Lures were cotton rolls, loaded with 5 mg of (2*R**,3*S**)-2,3-hexanediol in 1 ml of absolute ethanol, held in uncapped 3.7 ml glass vials, with controls treated only with 1 ml ethanol. Treatment and control traps were alternated along a transect of 14 traps.

Further bioassays were conducted in 2009 at field sites in Alameda County, CA, USA, where a population of *T. pilosicorne* was detected by light trapping. Traps were set at two sites: Rancho los Mochos Boy Scout Camp (RLM) and two areas on the grounds of the University of California's Lick Observatory (UCLO1 and UCLO2; Table 1). Bioassays were conducted fom 29 July to 8 August 2009. Pheromone lures consisted of low-density polyethylene sachets as described by Ray et al. (2011), loaded with 20 mg of (2*R**,3*S**)- or (2*R**,3*R**)-2,3-hexanediols in 1 ml of ethanol, or ethanol controls. Treatments and controls were replicated three times at each study site. Traps were as described above.

A further bioassay to identify the biologically active enantiomer for *T. pilosicorne* was conducted at UCLO1 and UCLO2 from 8–31 August 2009, using the same methods and numbers of replicates as the previous bioassay, but with lures loaded with (2*R**,3*S**)-, (2*R*,3*S*)-, or (2*S*,3*R*)-2,3-hexanediol (racemate, 50 mg in 1 ml of ethanol; enantiomers, 25 mg in 1 ml ethanol), or ethanol controls.

Bioassays conducted during the 2009 and 2010 seasons tested whether 2,3-hexanediols would also attract other *Tragosoma* species, in particular *T. depsarium* (L.). Potential field sites were identified by examining collection records in the Entomology Research Museum at the University of California, Riverside, the Essig Museum of Entomology at the University of California, Berkeley, and private insect collections. These new field sites included Barton Flats (BF), Blue Ridge (BR), and Lone Pine Canyon (LP) areas in southern California, and sites near Shafer Butte (SB) and Sage Hen Reservoir (SR) in Idaho (Table 1). Trap and lure designs were the same as those used in the study at UCLO (above), with the exception that traps were treated with a fluoropolymer dispersion emulsion (Fluon®; Graham et al., 2010).

Bioassays at BF and BR were conducted from 5 July to 17 August 2009 with traps baited with either the complete blend of all four 2,3-hexanediol isomers, (2*R**,3*R**)- or (2*R**,3*S**)-2,3-hexanediols, or a solvent control. At both sites, traps were set in three separate transects, with each transect containing one trap of each treatment.

Bioassays at SB and SR in Idaho were conducted 12–27 August 2009, as part of a larger survey testing attraction of cerambycid species to traps baited with a variety of candidate pheromones (unpublished data), but including (2*R**,3*R**)- and (2*R**,3*S**)-2,3-hexanediols, and a solvent control. Trap and lure designs were as described above, with each site containing a single replicate.

Bioassays in 2010 were conducted at LP and BF from 7 July to 23 August. The treatments were (2*R**,3*R**)-, (2*R**,3*S**)-2,3-hexanediols, and a solvent control. Trap and lure designs were as previously described, with two replicates used at each site.

A final bioassay was conducted at the BF site from 20 July to 9 September 2011, with two replicates, and treatments of (2*R*,3*R*)-, (2*S*,3*S*)-, (2*R*,3*S*)-, or (2*S*,3*R*)-2,3-hexanediols. Trap and lure designs were as previously described.

Statistical Analysis Differences among treatments were tested separately for each site and year, and blocked by date,

using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute, 2001). Differences between pairs of means were tested with the REGWQ means-separation test to control maximum experiment-wise error rates (PROC GLM; SAS Institute, 2001). We included in each analysis, only replicates that had a minimum number of specimens in order to assure sufficient replication for a robust analysis (at least 10 replicates; number of specimens ranged from 1–12, depending on total number captured).

Analysis of Headspace Volatiles Eight pupae of *T. depsarium* "sp. nov. Laplante" were collected on 3 June 2010 from downed *Pinus* sp. in the vicinity of the BF field site. Pupae were placed individually in artificial pupation chambers constructed of rolled newsprint (7 cm long×3 cm diam.) that were plugged with tissue paper, misted with water, and stored in a plastic bag at ambient temperature. Pupae were examined 2–3 times per week to monitor development. Adults eclosed from 26 June to 12 July 2010; teneral adults were returned to their paper tubes and temporarily stored at 4 °C until needed for experiments.

Headspace odors of individual adult *T. depsarium* "sp. nov. Laplante" (virgin-reared females, and both field-caught and virgin-reared males) were collected in an environmentally controlled room (26 °C, 65 % RH), using apparatus and methods previously described (Ray et al., 2011). Aerations were run for 4 d, after which the activated-charcoal collectors were extracted with dichloromethane (3 aliquots totaling 500 μl). Extracts were stored in a freezer (−4 °C) until analyzed.

To determine which 2,3-hexanediol stereoisomer(s) were produced by female *T. depsarium* "sp. nov. Laplante", an aliquot of headspace extract and samples of all four isomers were analyzed on a chiral stationary phase Cylodex B column (30 m×0.25 mm ID, 0.25 μ film; J&W Scientific, Folsom, CA, USA) with an oven-temperature program of 50 °C for 1 min, 3 °C.min^{-1} to 200 °C; injector temperature was 150 °C, and column head pressure 175 kPa. Under these conditions, the 2,3-hexanediol stereoisomers eluted in the following order: (2*S*,3*S*) 17.45 min, (2*R*,3*R*) 17.70 min, (2*R*,3*S*) 18.33 min, (2*S*,3*R*) 18.52 min.

Gas Chromatography/Electroantennography and Gas Chromatography/Mass Spectrometry Antennae from male beetles were used for coupled gas chromatography/electroantennogram detection assays (GC/EAD) of the 2,3-hexanediol stereoisomers, using specimens of *T. pilosicorne* captured in traps in 2009 (RLM field site), and *T. depsarium*

Table 2 Number of specimens of three species of *Tragosoma* captured in field bioassays of chiral and racemic 2,3-hexanediols

Species	Mean (± SE) number of males per trap									
Site (yr)	# individuals captured	2,3[a]	*R**,*R**	*R,R*	*S,S*	*R**,*S**	*R,S*	*S,R*	Blank	Fried.*Q*[b]
T. pilosicorne										
JMR (05)	12					**2.4±0.87a**			0b	8.0**
RLM (09)	56		0b			**8.0±2.4a**			0b	19.0***
UCLO (09)	137					**3.9±0.98a**	0b	**5.3±1.8a**	0b	40.8***
LPC (10)	23		0b			**3.29±1.3a**			0b	10.4**
T. depsarium "harrisi"										
BF (09)	13	0.14±0.14b	0b			**1.7±0.29a**			0b	24.3***
BF (10)	6		0b			**2.0±0.58a**			0b	7.62*
BF (11)	10			0b	0b		0.17±0.17b	**1.5±0.43a**		13.4**
SB, SR (09)	16		0b			**2.5±1.0a**			0.17b	10.3***
T. depsarium "sp. nov. Laplante"										
BF (09)	49	0b	**3.3±0.7a**			0b			0b	47.8***
BF (09)	74			**9.1±1.9a**	0.13±0.1b					12.3***
BR (09)	20	0.17±0.17b	**3.2±1.6a**			0b			0b	19.8***
SB, SR (09)	28		**6.75±2.9a**			0b			0b	12.0***
BF (10)	79		**19.8±5.9a**			0b			0b	10.5***
LPC (10)	33		**1.74±0.88a**			0b			0b	7.37*

Abbreviations for field sites as in Table 1. Means within species, sites, and dates with the same letters are not different (REGWQ means-separation test) at $P<0.05$; the greatest means are in bold

[a] Blend of (2*R**,3*R**)- and (2*R**,3*S**)-2,3-hexanediol

[b] Asterisks indicate a significant value of *Q*: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

"*harrisi*" and *T. depsarium* "sp. nov. Laplante" trapped in 2010 (LP and BF field sites). GC/EAD analyses were performed using DB-5 and DB-Wax columns (both 30 m× 0.25 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA, USA) with He as carrier gas. The GC was programmed from 40 °C for 1 min, 10 °C. min^{-1} to 275 °C for DB-5 and 250 °C for DB-Wax, and held for 45 min. Solvent extracts and solutions of standards (1 μl aliquots) were analyzed in splitless mode. The EAD apparatus and antennal preparations were as described in Ray et al. (2011).

Gas chromatography/mass spectrometry (GC/MS) analyses were carried out with an Agilent 6890N GC interfaced to a 5975C mass selective detector (Agilent, Santa Clara, CA, USA). The GC was fitted with an HP5-MS column (30 m× 0.25 mm i.d., 0.25 μm film), and the same temperature program and injection conditions as described above were used. Retention indices (RI) were calculated relative to blends of straight-chain hydrocarbons. For increased precision, KI values on the DB-Wax column were obtained using a GC oven program rate of 5 °C rather than 10 °C.min^{-1}.

Scanning Electron Microscopy Scanning electron microscopy was used to examine the prothoraces of *T. pilosicorne* adults for the presence of pores that are associated with the production of 2,3-hexanediol pheromones in cerambycine species (e.g., Ray et al., 2006). The male specimen had been collected during bioassays in 2005 (see above), while the female specimen was donated by the Entomology Research Museum University of California, Riverside (no collection data available; identifications confirmed using the characters of Linsley, 1962). Specimens were prepared following the methods of Ray et al. (2006), and were imaged with an environmental scanning electron microscope equipped with a field-emission electron gun (Philips XL30, FEI Company, Hillsboro, OR, USA) at 5.0 kV. Remnants of specimens have been retained by AMR at Xavier University and scanning electron micrographs have been submitted to Morphbank (www.morphbank.net, image numbers 549802–549806).

Voucher specimens of all three *Tragosoma* species have been submitted to the Entomology Research Museum (UC Riverside, Riverside, CA, USA) with the following identification codes: *T. pilosicorne* (from study site JMR) UCRC ENT 291760-81; *T. pilosicorne* (UCLO, RLM) UCRC ENT 301599-607; *T. depsarium "harrisi"* (SB) UCRC ENT 301608-611; *T. depsarium* "sp. nov. Laplante" (SB, BF) UCRC ENT 301612-620.

Results and Discussion

During a preliminary field-screening bioassay conducted in 2005, four male *T. pilosicorne* were caught in two traps baited with (2*R**,3*S**)-2,3-hexanediol, whereas no *T. pilosicorne* were caught in any of the other traps. In a follow-up bioassay at the JMR site, 12 male *T. pilosicorne* were captured, all in traps baited with (2*R**,3*S**)-2,3-hexanediol (treatment different than control, Table 2). Attraction to that racemic blend was confirmed by subsequent trials in 2009 and 2010 at three other field sites, with the active enantiomer determined as (2*S*,3*R*)-

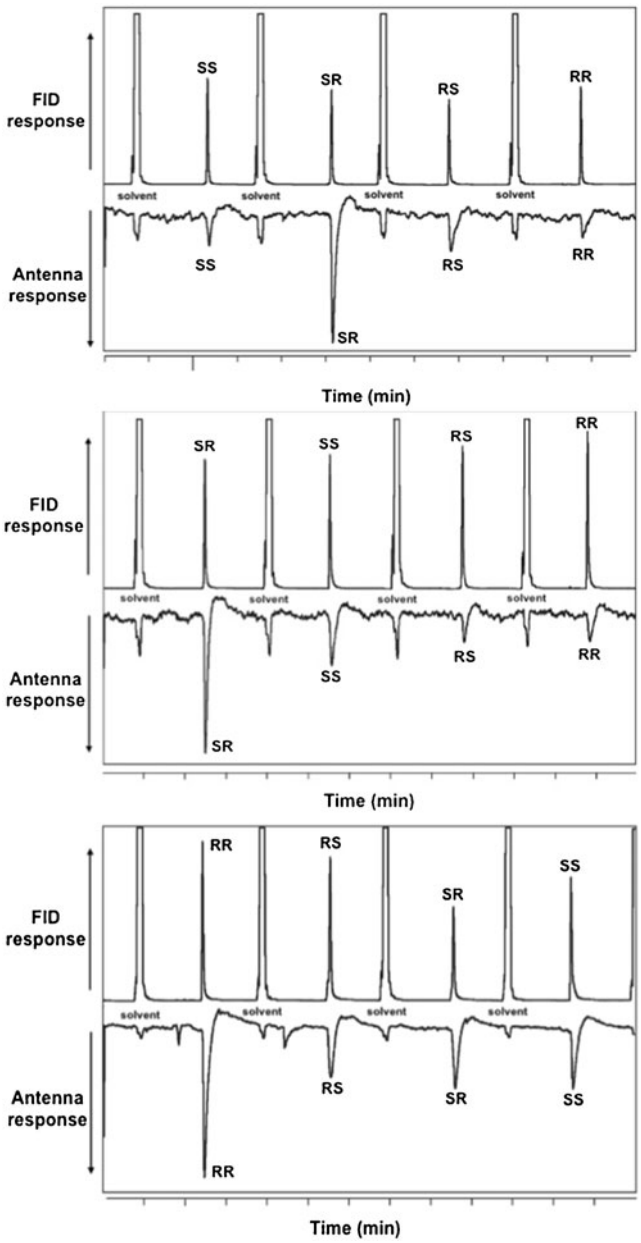

Fig. 1 Representative gas chromatography/electroantennogram detection traces showing responses to stereoisomers of 2,3-hexanediols by antennae of male *Tragosoma pilosicorne* (*top*), *T. depsarium* "*harrisi*" (*middle*), and *T. depsarium* "sp. nov. Laplante" (*bottom*). In each frame, the top trace shows the GC peaks (flame ionization detection; FID) from sequential injection of the stereoisomers in random order on a DB-5 column (30 m×0.25 mm×0.25 micron film, 100 °C isothermal), and the inverted, bottom trace shows the antennal response

2,3-hexanediol (Table 2). The single enantiomer (2*S*,3*R*)-2,3-hexanediol and racemic (2*R**,3*S**)-2,3-hexanediol were equally attractive in the 2009 trial, indicating that the unnatural enantiomer, (2*R*,3*S*)-2,3-hexanediol, did not inhibit attraction of male beetles. Also, antennae of male *T. pilosicorne* responded most strongly to the (2*S*,3*R*)-2,3-enantiomer, consistent with it being a sex pheromone component. The other enantiomers elicited detectable, but lower, responses from antennae (Fig. 1). This suggests some interaction between the unnatural stereoisomers and the olfactory receptors, as has been shown to occur with chiral pheromone components of other cerambycid species (e.g., Mitchell et al., 2012). However, the lower responses also may have been due, in part, to contamination of the chiral standards with trace amounts of other stereoisomers.

Specimens of *T. depsarium* captured at Barton Flats in 2009 appeared to represent two distinct morphologies that were consistently associated with different trap treatments. We subsequently discovered that these two 'morpho-species' had been recognized by S. Laplante. In the present article, these species are designated as *T. depsarium* "*harrisi*" (antennae gracile and barely attaining elytral apex, integument of elytra medium brown, pronotum clothed in dense, golden setae) and *T. depsarium* "sp. nov. Laplante" (antennae robust and attaining or exceeding elytral apex, integument of elytra dark brown, setae on pronotum less dense [AMR, pers. obs.]).

As was the case with *T. pilosicorne*, male *T. depsarium* "*harrisi*" were attracted to (2*R**,3*S**)-2,3-hexanediol (Table 2), with subsequent bioassays confirming that males were specifically attracted to (2*S*,3*R*)-2,3-hexanediol. This stereoisomer also elicited stronger responses from antennae of males in GC/EAD analyses than did the other three stereoisomers (Fig. 1), further suggesting that it may be a sex pheromone component of this species.

Male *T. depsarium* "sp. nov. Laplante" differed from the other two species in being attracted consistently to racemic (2*R**,3*R**)-2,3-hexanediol, and tests with the two enantiomers showed that males were specifically attracted to the (2*R*,3*R*)-2,3-hexanediol enantiomer (Table 2). Although no direct comparison was made between (2*R**,3*R**)-2,3-hexanediol and (2*R*,3*R*)-2,3-hexanediol, the fact that the racemate attracted significant numbers of beetles in several different bioassays suggests that the (2*S*,3*S*)-2,3-hexanediol enantiomer was not inhibitory. In contrast, the mixture of all four stereoisomers of 2,3-hexanediol attracted very few males (Table 2), indicating that (2*R*,3*S*)- and/or (2*S*,3*R*)-2,3-hexanediol inhibited attraction. Antennae of male *T. depsarium* "sp. nov. Laplante" responded most strongly to (2*R*,3*R*)-2,3-hexanediol in GC/EAD assays (Fig. 1), consistent with it being the most attractive enantiomer.

Identification of the pheromones produced by females of *Tragosoma* species was hindered by the difficulty in obtaining females. Attempts to capture females by light trapping at the RLM, UCLO, and BF field sites during the known activity periods of the adults in 2009 were unsuccessful. However, eight pupae of *T. depsarium* "sp. nov. Laplante" were obtained from infested logs, and the adults that emerged were used for collection of headspace volatiles. The volatiles collected from female *T. depsarium* "sp. nov. Laplante" contained one major sex-specific peak, confirmed as (2*R**,3*R**)-2,3-hexanediol by retention time (DB-5) and mass spectral matches with those of a standard. On this column, we achieved baseline separation of the (2*R**,3*R**)- and (2*R**,3*S**)-diastereomers. Further analysis on a chiral stationary phase Cyclodex B GC column, comparing the retention time of the insect-produced compound to those of all four 2,3-hexanediol stereoisomers, further confirmed the identification, and revealed that the insect produced (2*R*,3*R*)-2,3-hexanediol exclusively. Analogous extracts of headspace volatiles of male *T. depsarium* "sp. nov. Laplante" did not show this compound, indicating that production of (2*R*,3*R*)-2,3-hexanediol was female-specific.

Scanning electron microscopy revealed that female *T. pilosicorne* had numerous pores on the ventral side of the lateral spine of the prothorax that were entirely absent in males (Fig. 2). Similar pores on the prothoraces of female *T. depsarium "harrisi"* and *T. depsarium* "sp. nov. Laplante" were located by light microscopy. This sexual dimorphism is analogous to that of cerambycine species that produce pheromones comprised of 2,3-alkanediols and related 3-

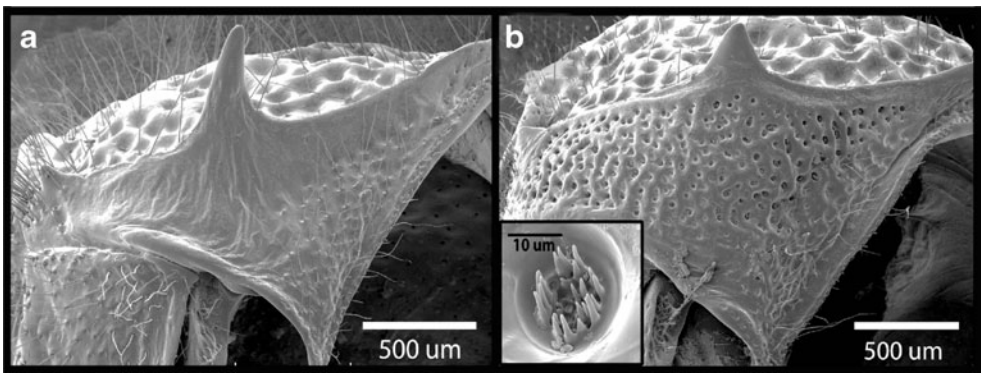

Fig. 2 Scanning electron micrographs of prothoraces of adult male (**a**) and female (**b**) *Tragosoma pilosicorne*. Prothoraces of females have pores lying within pits (see inset) that are absent in males

hydroxyalkan-2-ones; however, in the cerambycines it is the males that produce volatile pheromones and have the prothoracic pheromone gland pores (Hanks et al., 2007; Lacey et al., 2007; Ray et al., 2009). This suggests that there may have been a switch in the sex that produces pheromone between the *Tragosoma* spp. and the cerambycines, but the pheromone chemistry and the pheromone production sites appear to have remained unchanged.

In summary, this study indicates that the female-produced sex attractant pheromone for *Tragosoma depsarium* "sp. nov. Laplante" is (2*R*,3*R*)-2,3-hexanediol, and provides evidence from field bioassays and electroantennography that (2*S*,3*R*)-2,3-hexanediol may be a sex pheromone component of the congeners *T. pilosicorne* and *T. depsarium* "*harrisi*". These experiments suggest a second shared pheromone motif within the cerambycid subfamily Prioninae. Whereas the available evidence suggests that female beetles in the prionine genus *Prionus* produce similar, or perhaps identical, pheromones (Barbour et al., 2011), the results described above suggest that females of at least two species of *Tragosoma* may share a pheromone structure, whereas a third species may use a diastereomer of the same basic structure as its pheromone. Remarkably, the same compounds are being used as pheromones by species from two different cerambycid subfamilies, as well as by different sexes. These facts suggest a close phylogenetic relationship between the two subfamilies, which indeed has been suggested in a recent phylogenetic analysis (Sykorova, 2008). The results from the three *Tragosoma* species also suggest that cerambycids take advantage of available stereoisomers to create unique pheromone channels, thus minimizing the possibility of cross-attraction. However, host plant volatiles, minor components of the insect-produced blends, or contact pheromones may also play important roles in maintaining reproductive isolation, particularly among sympatric species.

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RAPID COMMUNICATION

Sex Pheromone of the Smaller Clearwing Moth *Synanthedon tenuis* (Butler)

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Abstract The smaller clearwing moth, *Synanthedon tenuis* (Butler) (Lepidoptera: Sesiidae), is a major pest of persimmon in northeast Asia. A previous study reported attraction of *S. tenuis* males to *Z*3,*Z*13-18:OAc, but this compound had no effect on male catch in the persimmon orchards in Korea. In this study, we analyzed pheromone gland extracts of *S. tenuis* females and identified *Z*3,*Z*13-18:OH as the main component. In field trapping trial, *Z*3,*Z*13-18:OH alone was attractive to *S. tenuis* males and competitive with live virgin females. These results indicate that the pheromone of this species consists of a single component, *Z*3, *Z*13-18:OH. However, *Z*3,*Z*13-18:OAc, a previously reported attractant, was not detected in the gland extracts of females. Furthermore, the addition of *Z*3,*Z*13-18:OAc to the main pheromone component strongly inhibited attraction for males, suggesting that the diene acetate is not a pheromone component. This is the first report of an octadecadienol as female-produced sex pheromone from the genus *Synanthedon*.

Keywords *Synanthedon tenuis* · Sex pheromone · Lepidoptera · Sesiidae

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Introduction

The smaller clearwing moth, *Synanthedon tenuis* (Butler) (Lepidoptera: Sesiidae), is one of the major pests of persimmon, *Diospyros kaki* Thunb (Ebenaceae) in Korea, China, and Japan (Arita et al., 2004). Larvae bore into and feed on young twigs and stems, causing considerable damage. This pest is difficult to control with insecticides because of the internal feeding habit of larvae. Thus, pheromone traps would be a useful tool for monitoring in order to optimize timing of control measures (e.g., Bergh et al., 2009). Moreover, a synthetic sex pheromone could lead to an environmentally safe control system based upon mating disruption or mass trapping (e.g., Leskey et al., 2009).

A previous field trial conducted in Japan showed that *S. tenuis* males were attracted to *Z*3,*Z*13-18:OAc (Tamaki et al., 1977). Based on this result, flight activity of the Korean population of *S. tenuis* was monitored with traps baited with a lure containing *Z*3,*Z*13-18:OAc in the persimmon orchards. However, these monitoring traps were not effective in catching *S. tenuis* males (C. Y. Yang, personal observation). Therefore, we decided to examine the sex pheromone communication system of the Korean population of *S. tenuis* by chemical analyses of the pheromone glands of females and field-testing of pheromone blends.

Methods and Materials

Insects During the summer of 2010 and 2011, late-instar larvae of *S. tenuis* were collected from persimmon trees at Gimhae (35.1°N, 128.5°E), Korea. Larvae were reared on persimmon branches in screen cages (30×30×30 cm), and maintained at 23 °C under a L14:D10 photoperiod. Emerging adults were collected each day, and the sexes were separated according to the presence of distinctive hair tufts (Arita et al., 2004). Female moths were kept individually in

plastic bottles (7 cm high and 2.5 cm diam) and provided with a cotton pad soaked with a 10 % sucrose solution as food.

Chemicals Synthetic pheromone components were obtained from Pherobank (Wageningen, The Netherlands; >99 % purity). Dimethyldisulfide (DMDS), iodine, and sodium thiosulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pheromone Extraction The terminal abdominal segments including the pheromone gland of 1- to 2-d-old females were excised during the 7th through 9th hour of the photophase, the peak time of calling, and extracted in a 0.3-ml conical glass vial (Wheaton, Millville, NJ, USA) containing hexane (10 μl per pheromone gland) for 10 min. The hexane extract was transferred into another vial and stored at −20 °C until analysis.

Chemical Analysis Gland extracts and synthetic standards were analyzed on an Agilent 6890N gas chromatograph (GC) interfaced to an Agilent 5975C mass-selective (MS) detector. The GC was equipped with a DB-5MS column or DB-23 column (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Injector and transfer line temperatures were 250 °C. The ionization voltage was 70 eV, and the ion source temperature was 230 °C. Samples were injected splitless, and the split valve was opened 0.75 min after manual injection. Helium was the carrier gas (1 ml/min). For the DB-5MS column, the GC oven temperature was programmed from 80 °C (1-min hold) to 220 °C at 10 °C/min, and held for 10 min. For the DB-23 column, the oven temperature was maintained at 80 °C for 1 min, increased to 180 °C at 10 °C/min, then to 220 °C at 5 °C/min, and held at the final temperature for 10 min. Compounds from gland extracts were identified by comparison of their retention times and mass spectra with those of authentic standards on both columns.

Dimethyldisulfide Derivatization Determination of double-bond positions in unsaturated compounds of the pheromone extract was accomplished by reaction with DMDS (Buser et al., 1983). DMDS derivatization of a 24-female gland extract was performed as previously described by Yang et al. (2011a). The DMDS adduct was analyzed with a DB-5MS column, using a temperature program of 100 °C to 300 °C at 10 °C/min with other GC conditions as those described above.

Field Tests Field trials conducted in the persimmon orchards in Gimhae, Korea. Two replicated field experiments were carried out from 30 May to 4 June, and from 29 August to 3 September 2011 to compare the attraction of *S. tenuis* males to *Z*3,*Z*13-18:OH alone and in combinations with other structurally related compounds, *E*2,*Z*13-18:OAc, *Z*2,*Z*13-18:OAc, *E*3,*Z*13-18:OAc, *Z*3,*Z*13-18:OAc, *E*2,*Z*13-18:OH, *Z*2,*Z*13-18:OH, and *E*3,*Z*13-18:OH. Sticky delta traps (Green Agro Tech, Gyeongsan, Korea) were baited with white rubber septa (8 mm OD; Aldrich Chemical, Milwaukee, WI, USA) loaded with test compounds in hexane. Unbaited traps were used as negative controls in both trials, and traps baited with a single 0- to 1-d-old virgin female in small mesh cages were used as positive controls in the second trial. Traps were suspended from persimmon branches 1.0 to 1.5 m above the ground. Traps were deployed in a complete randomized block design with three replicate blocks. Traps were placed approximately 10 m apart within a block, and blocks separated by at least 100 m. Captured males were counted and removed every 2–3 d. Sticky inserts and virgin females were replaced at each count.

Capture data (x) were transformed to log ($x+1$) and submitted to one-way analysis of variance (ANOVA). Treatments that failed to capture males were not included in the analyses to avoid violating assumptions of ANOVA. Means were compared by Tukey's test at $\alpha=0.05$ (SAS Institute Inc., 2010).

Results and Discussion

GC-MS analyses on DB-5MS and DB-23 columns of gland extracts of female *S. tenuis* indicated the presence of one pheromone-related compound (compound 1) along with straight-chain alkanes frequently found in lepidopteran glands (Fig. 1). The mass spectrum of compound 1 showed a molecular ion *m/z* 266, and diagnostic fragment ions *m/z* 248 (M-18), 222, and 194, suggesting an octadecadienol. Analysis of the DMDS-derivative revealed an adduct of C_{18} dien-1-ol with a molecular ion at *m/z* 454 (266+2MeSSMe) and diagnostic ions at *m/z* 301, 289, 117 ($C_5H_{10}SMe$), and 105 (C_3H_5OHSMe) indicative of an octadecadienol with the double bonds at the 3- and 13- positions (Francke et al., 2004). The retention times of compound 1 on both columns coincided with those of synthetic *Z*3,*Z*13-18:OH (DB-5MS: 17.40 min, DB-23: 15.71 min), but not with its geometrical isomers. Accordingly, the structure of diene present in the pheromone glands was determined to be *Z*3,*Z*13-18:OH.

A total of 77 *S. tenuis* males were captured in field trial 1. *Z*3,*Z*13-18:OH as a single component was attractive to males, whereas *Z*3,*Z*13-18:OAc was unattractive alone (Table 1). Trap catches of males to *Z*3,*Z*13-18:OH were unaffected by the addition of *E*2,*Z*13-18:OAc, *Z*2,*Z*13-18:OAc, *E*3,*Z*13-18:OAc, *E*2,*Z*13-18:OH, or *Z*2,*Z*13-18:OH. In contrast, addition of 10 % *Z*3,*Z*13-18:OAc or *E*3,*Z*13-18:OH to *Z*3,*Z*13-18:OH strongly inhibited attraction of males. In field trial 2, traps captured a total of 109 *S. tenuis* males. This trial again showed that the seven minor components did not increase attraction of male moths to *Z*3,*Z*13-18:OH. Traps baited with 500 μg of *Z*3,

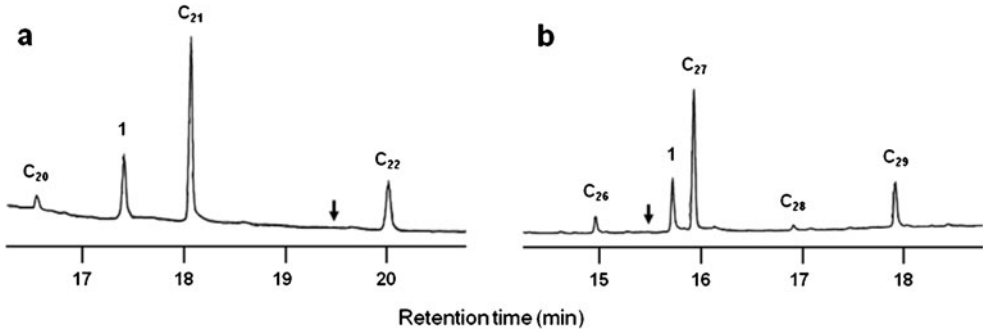

Fig. 1 Representative total ion chromatograms of GC-MS analysis of pheromone gland extracts from female *Synanthedon tenuis* on a DB-5MS column (**a**) and DB-23 column (**b**). Compound identities as follows: 1) *Z*3,*Z*13-18:OH, C_{20} eicosane, C_{21} heneicosane, C_{22} docosane, C_{26} hexacosane, C_{27} heptacosane, C_{28} octacosane, C_{29} nonacosane. Arrows indicate the retention times of synthetic *Z*3,*Z*13-18:OAc on each column

*Z*13-18:OH captured more males than did the caged virgin females, although the difference was not statistically significant.

Based on the chemical analyses and the results of the field trials, we have identified the sex pheromone of *S. tenuis* as *Z*3, *Z*13-18:OH. No additional compounds having pheromone-type structures were detected in the gland extracts (ca. 100 pg of detection limit), and none of the tested compounds had synergistic effects on trap catches of *S. tenuis* males. Furthermore, *Z*3,*Z*13-18:OH alone was as attractive as virgin female moths, and no other moth species were attracted to this compound over the course of the trials, suggesting that *Z*3,*Z*13-18:OH by itself is an effective attractant for monitoring *S. tenuis*.

*Z*3,*Z*13-18:OH had been previously reported as a sex pheromone component for other sesiid species, including *Glossosphecia romanovi*, *Nokona pernix*, *Paranthrene robiniae*, *Paranthrene tabaniformis*, *Sesia apiformis*, *Sesia siningensis*, and *Ichneumonoptera chrysophanes* (El-Sayed, 2012). In addition, *Z*3,*Z*13-18:OH had been previously discovered to be a constituent of attractants for five species of the genus *Synanthedon* (*S. acerni*, *S. decipiens*, *S. geliformis*, *S. rileyana*, and *S. sequoia*), but has not been identified from their conspecific females. To our knowledge, this is the first report of *Z*3,*Z*13-18:OH as a sex pheromone component in the genus *Synanthedon*.

As noted above, the addition of *Z*3,*Z*13-18:OAc or *E*3, *Z*13-18:OH to the main pheromone component, *Z*3,*Z*13-18:OH, completely inhibited attraction for *S. tenuis* males. This indicates that *S. tenuis* males would not be attracted to females of sympatric sesiid species, *G. romanovi* (Yang et al., 2011a), *Synanthedon haitangvora* (Yang et al., 2009), or *Synanthedon bicingulata* (Yang et al., 2011b) that emit *Z*3, *Z*13-18:OAc as one of their sex pheromones. Similarly, *E*3, *Z*13-18:OH could play a role in pheromone communication between sympatric *S. tenuis* and *Nokona regalis* because this diene alcohol is a pheromone component of the latter species (Guo et al., 1990).

In an earlier field test, Tamaki et al. (1977) found that *Z*3, *Z*13-18:OAc alone was attractive to male *S. tenuis* in Japanese persimmon orchards. However, we found no evidence

Table 1 Catches of male *Synanthedon tenuis* in traps baited with *Z*3,*Z*13-18:OH alone and in combination with different minor components

Treatment	Catch per trap (mean ± SE)[a]	
	Trial 1[b]	Trial 2[c]
*Z*3,*Z*13-18:OH (500 μg)	3.3±0.3a	5.3±1.5a
*Z*3,*Z*13-18:OH (500 μg) + *E*2,*Z*13-18:OAc (50 μg)	4.3±0.7a	7.0±2.1a
*Z*3,*Z*13-18:OH (500 μg) + *Z*2,*Z*13-18:OAc (50 μg)	3.7±0.9a	5.0±1.2a
*Z*3,*Z*13-18:OH (500 μg) + *E*3,*Z*13-18:OAc (50 μg)	4.7±0.9a	6.3±1.8a
*Z*3,*Z*13-18:OH (500 μg) + *Z*3,*Z*13-18:OAc (50 μg)	0	0
*Z*3,*Z*13-18:OH (500 μg) + *E*2,*Z*13-18:OH (50 μg)	5.3±1.5a	4.7±1.2a
*Z*3,*Z*13-18:OH (500 μg) + *Z*2,*Z*13-18:OH (50 μg)	4.3±0.3a	4.3±0.9a
*Z*3,*Z*13-18:OH (500 μg) + *E*3,*Z*13-18:OH (50 μg)	0	0
*Z*3,*Z*13-18:OAc (500 μg)	0	0
Virgin female	–[d]	3.7±3.2a
Blank	0	0

[a]Means in each trial followed by the same letter are not significantly different at $P<0.05$ by Tukey's test

[b]Trial 1 conducted in persimmon orchards at Gimhae, Korea from 30 May to 4 June 2011

[c]Trial 2 conducted in persimmon orchards at Gimhae, Korea from 29 August to 3 September 2011

[d]Not tested

that females from Korean population of *S. tenuis* produced *Z*3,*Z*13-18:OAc (Fig. 1). As shown in Table 1, pure *Z*3,*Z*13-18:OAc caught no male moths at all. Moreover, attraction of males to their pheromone was strongly antagonized by addition of 10 % *Z*3,*Z*13-18:OAc. A morphological investigation of the genitalia of *S. tenuis* has revealed no differences between Korean and Japanese populations (Yang-Seop Bae, personal communication). Therefore, further research is required to clarify the situation, including investigation of female pheromone production from Japan to China.

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Identification and Binding Characterization of Three Odorant Binding Proteins and One Chemosensory Protein from *Apolygus lucorum* (Meyer-Dur)

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Abstract For the sucking insect, *Apolygus lucorum,* taste is essential for finding host plants and oviposition sites. In *A. lucorum,* taste relies largely on the sensory system located within its proboscis. In this study, we constructed a cDNA library from *A. lucorum* proboscises and conducted preliminary analysis of 1554 ESTs. From this collection, we identified three putative odorant-binding proteins (AlucOBP3, AlucOBP4, AlucOBP6) and one chemosensory protein (AlucCSP1). Quantitative real-time polymerase chain reaction (qPCR) was used to study the expression pattern of these four genes. All four were expressed mainly in antennae, proboscises and legs, suggestive of roles in olfaction and gustation. We expressed and purified recombinant versions of AlucOBP3, AlucOBP4, AlucOBP6, and AlucCSP1 in a prokaryotic expression system. The ligand-binding specificities of the four proteins then were investigated in competition assays using 4,4′-dianilino-1,1′-binaphthyl-5, 5′-sulfonic acid (bis-ANS) as a probe. Of the 58 small organic compounds and five cotton secondary metabolites tested, plant volatiles cannot effectively displace bis-ANS from any of the four proteins. In contrast, most of the cotton secondary metabolites have high affinities for the three OBPs and AlucCSP1, indicating that these binding proteins more likely play a role in gustation than in olfaction.

Keywords *Apolygus lucorum* · Odorant binding protein (OBP) · Chemosensory protein (CSP) · Tissue-specific expression · Binding specificity · Hemiptera · Miridae

Introduction

The detection of chemicals plays an important role in insect survival and reproduction. Insects sense their environment by using sensitive olfactory and gustatory systems to react with appropriate behavioral responses (Hildebrand and Shepherd, 1997; Krieger and Breer, 1999; Field et al., 2000). *Drosophila* and many other insects detect tastants on multiple body parts through hair-like projections referred to as gustatory sensilla (Stocker, 1994; Singh, 1997). Insect gustatory sensilla mainly occur on the mouthparts, cercopod, antennae, tarsi, and female ovipositor. There also are a small number of gustatory sensilla on wings of many insects. The proboscis of insects contains high concentrations of gustatory sensilla both externally and internally, and therefore, might be considered the functional equivalent of the mammalian tongue (Montell, 2009). Chemicals enter sensilla through holes in the epidermis and are then bound by odorant binding proteins (OBPs) or chemosensory proteins (CSPs). These complexes then are transported through the water-soluble lymph within the sensilla to receptors located on the dendrites of the sensory neurons (Pelosi et al., 2005; Zhou et al., 2009).

The mouthparts of plant bugs are characterized by various sensory structures that come into contact with the plant substrate either during the search for food or when the female looks for a suitable oviposition site. There are 11–12 uniporous gustatory sensilla that are innervated by 3–6 sensory neurons present on the tip of the labium of *Lygus rugulipennis* females, which play a role in assessing the suitability of the substrate as a food source (Romani et al., 2005). Plant

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Table 1 Oligonucleotide primers used for gene expression analysis of the odorant binding proteins and chemosensory protein from *Apolygus lucorum*

Primer name	Sequence (5′-3′)	Position (bp)
OBP3-Forward	TGGTGGTTGACGGGAAGGT	221–239
OBP3-Reverse	TCATCATCCCATTTCTCCTTGTT	265–287
OBP3-Probe	FAM-ATGCTTTCCATGCGTCGGCGTC-Eclipse	241–262
OBP4-Forward	TGATAGTGTCGTTGAGGATTTTATCG	132–157
OBP4-Reverse	CTTCCATATAACATGCGACCATACA	193–217
OBP4-Probe	FAM-AGGTACAACAAGCCACAATCTGAATCCGG-Eclipse	160–188
OBP6-Forward	ATGTTATCGCCGTGCAAGCT	275–294
OBP6-Reverse	CTTCCGGAGGCACTGTATCTG	362–382
OBP6-Probe	FAM-TGAAATAACCAAGGCAAAGGAAATCATCCA-Eclipse	325–353
CSP1-Forward	GAACACGCGGCTCTACAAGAA	106–125
CSP1-Reverse	TCTTTTCCATCAGGAGTGCATTT	154–176
CSP1-Probe	FAM-TTCGACTGCCTCGCCAACAAGGG-Eclipse	130–153
18S -Forward	GGCGACTTATCCTTCAAATGC	162–182
18S -Reverse	GATGTGGTAGCCGTTTCTCAG	272–292
18S -Probe	FAM- CATGGTTGTTACGGGTAACGGGGAA -Eclipse	221–245

secondary substances play a major role in feeding or ovipositing stimulants for some specialist insects (Elsayed, 2011).

Apolygus lucorum is an important species of plant bug, with more than 100 described host plants throughout China. Since 1997, commercial plantings of Bt cotton in China have effectively controlled *Lepidopteran* pests (such as cotton bollworms) and reduced the use of pesticides, resulting in sucking insects becoming dominant pests. The population numbers of plant bugs have gradually increased by 15–30 % (or even 50–70 %), resulting in significant damage of cotton (Lu et al., 2010). Because the plant bugs found on cotton have a broad host range, a rapid rate of reproduction, few natural enemies, and are highly mobile, biological control is not an ideal control strategy. In addition, large-scale use of chemical pesticides may cause resistance and environmental pollution. To elucidate the molecular mechanism of *A. lucorum* gustation and to design novel intervention strategies against these plant bugs base on gustatory sensilla, we constructed a cDNA library from proboscises of *A. lucorum* and successfully identified three OBP and one CSP gene. Quantitative real-time polymerase chain reaction (qPCR) was used to determine the expression patterns of the fours genes. Finally, volatile chemicals and cotton secondary metabolites were used to study the binding characteristics.

Table 2 Oligonucleotide primers used for bacterial expression of odorant binding proteins and chemosensory protein from *Apolygus lucorum*

Primer name	Sequence (5′-3′)
OBP3-Forward	cggatccGTCAGCAAAGAATACCACGAT[a]
OBP3-Reverse	ggaattcTTATCGCCTTTTTGGGTGTTC
OBP4-Forward	cggatccGTGATCAATAAGGACTATTT
OBP4-Reverse	ccgctcgagTCAGTGCTTGAGCTGCGCAA
OBP6-Forward	ggatccAAGGAATTAACAGACGAGCAGAAGG
OBP6-Reverse	ggaattcTCAGGGCCCCCCCGAAA
CSP1-Forward	cggatccGCTGCCACGTACACGTCCAA
CSP1-Reverse	ggaattcTCAGTGGCTTCCGGGCAATT

[a] Restriction enzyme sites for *Bam*HI(ggattc), *Eco*RI (gaatcc), and *Sal*I (gtcgac) are underlined

Methods and Materials

Insects Apolygus lucorum was bred at the Cotton Research Institute, Chinese Academy of Agricultural Sciences (Henan, China). Insects were fed with fresh beans and 10 % honey in a crisper at 29±1 °C, 60 %±5 % (relative humidity), 14L:10D. A proboscis cDNA library was created from the proboscises of approximately 150 3-d-old adult *A. lucorum*. Quantitative real-time polymerase chain reaction (qPCR) was performed using the reverse transcribed RNA isolated from heads (without antennae and proboscises), thoraxes, abdomen, legs, wings, antennae, and proboscises of 3-d-old adult *A. lucorum*.

RNA Extraction and cDNA Library Construction Total RNA was extracted using Trizol (Invitrogen Corp., Carlsbad, CA, USA) according to instructions, and examined under UV light after electrophoresis in 2 % agarose. Double-stranded cDNA was prepared using the SMART™ PCR cDNA Synthesis Kit (Clontech, Cambridge, UK). In brief, SMART CDS Primer II A (12 μM), SMARTerII oligonucleotide (12 μM) and 1 μg total RNA were used for reverse transcription to synthesize the first strand cDNA. Then, PCR Primer II A (12 μM) and SMART CDS Primer II A (12 μM) were used to amplify the second strand by long-distance PCR (LD-PCR)

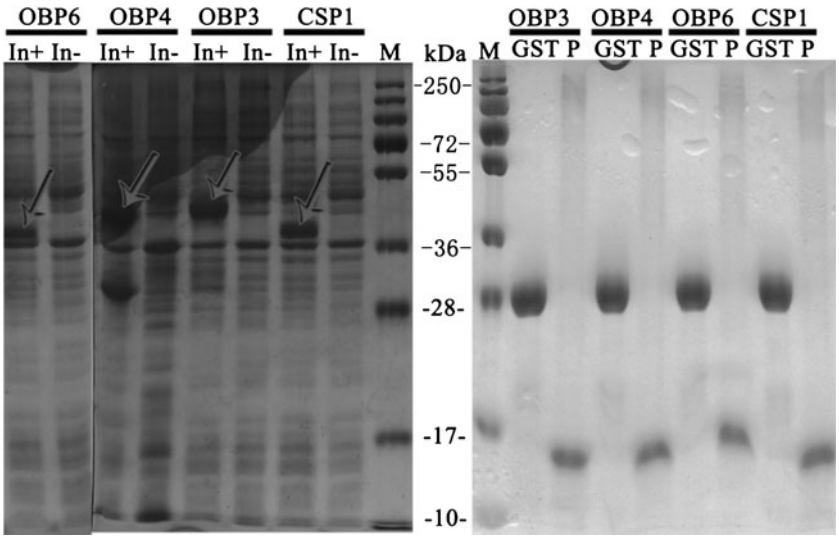

Fig. 1 SDS-PAGE analysis of prokaryotic-expressed and purified *Apolygus lucorum* odorant-binding proteins and chemosensory protein. M: protein molecular marker; In+: induction with IPTG; In-: before induction with IPTG. The target bands are indicated with *arrows*

to synthesize double-stranded cDNA. The synthesized products were separated by electrophoresis on a 1.2 % agarose gel and purified with a CHROMA SPIN-400 column, then ligated into the vector and transformed into DH5α *E. coli* cells (TaKaRa, Dalian, Liaoning, China). Transformed colonies were selected on ampicillin plates.

For qPCR, total RNA was isolation using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA). The PrimeScript ® RT reagent kit (Perfect Real Time) (TaKaRa) was used for reverse transcription.

Clone Sequencing and Sequence Analysis Clones containing a cDNA insert greater than 400 bp were sequenced using an ABI3730 sequencer. All high quality EST sequences were assembled as unigenes using VectorNTI. Unigene sequences were subjected to BLASTX, TBLASTX, and BLASTN searches (Altschul et al., 1998), with a cut-off E-value of 1.0E-5 used (Anderson and Brass, 1998). Finally, the Blast2 GO program was used to classify EST sequences by cellular component, function, metabolic process, and species similarity (http://www.geneontology.org).

Quantitative Real-time PCR and Data Analysis TaqMan probes were used for qRT-PCR experiments. TaqMan probes and primers were designed and synthesized by Takara from the sequences of OBPs and CSP (Table 1). A 20 μl qPCR mix contained 10 μl of Premix ExTaq (2×), 0.4 μL of each primer (10 μmol/L), 0.8 μl of probe (10 μmol/L), and 1 μl sample cDNA and 7.4 μl sterilized ultrapure H_2O. Negative controls were non-template reactions (replacing cDNA with H_2O). Cycling parameters were 95 °C for 3 min, 40 cycles of 95 °C for 5 sec, and 60 °C for 20 sec. Reactions were performed in a Rotor-Gene3000 quantitative PCR instrument (Corbett Research, Sydney, Australia). A ten-fold dilution series were used to construct a standard curve to determine the PCR efficiencies and for downstream quantification analysis. In all experiments, all primers gave amplification efficiencies of 90–100 %. Each reaction was run in triplicate (technical replicate). C_T values were collected after completion of the reaction, and data analysis was performed using $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001). The relative expression levels of OBPs and CSPs in different tissues were quantified with the endogenous reference gene 18S rRNA (GU194612). To enable the same amplification efficiency of cDNAs from different tissues, preliminary experiments were performed, and the cDNA template was diluted for reverse transcription (Zhang et al., 2009).

Recombinant Protein Expression and Purification Recombinant forms of *A. lucorum* OBPs and CSP were obtained by

Table 3 Properties of odorant binding proteins and chemosensory protein from *Apolygus lucorum*

Gene name	ORF (bp)	Length (aa)	SignalP (aa)	Accession number	ESTs (#)	Homology search with known proteins				
						Score	*E*-value	%Identify	Species	Protein ID
AlucOBP3	453	150	19	JN573222	65	306	1.00E-106	95 %	*A.lin*	ACZ58082.1
AlucOBP4	459	152	20	JN573223	53	308	3.00E-107	98 %	*A.lin*	ACZ58079.1
AlucOBP6	462	153	27	JN573224	99	296	2.00E-101	98 %	*A.lin*	ACZ58032.1
AlucCSP1	393	130	17	JN573217	20	149	4.00E-45	60 %	*S.cal*	ADG96053.1

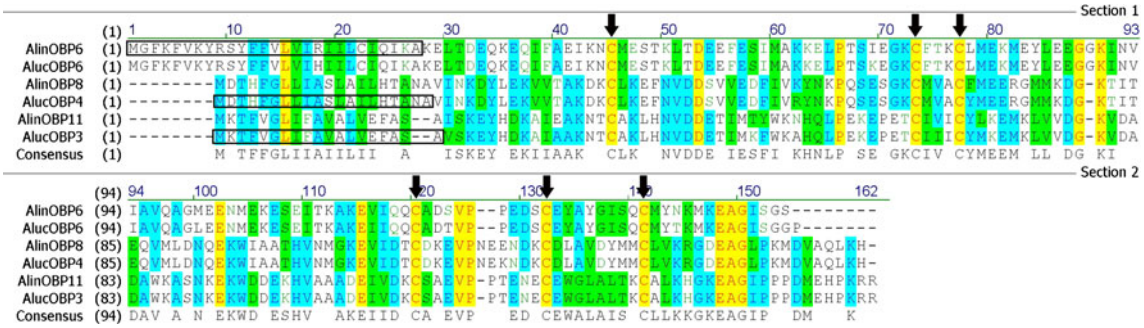

Fig. 2 Alignment of the *Apolygus lucorum* odorant-binding proteins and *Adelphocoris lineolatus* odorant-binding proteins. Predicted signal peptides are boxed. Conserved cysteine residues are indicated with *arrows*

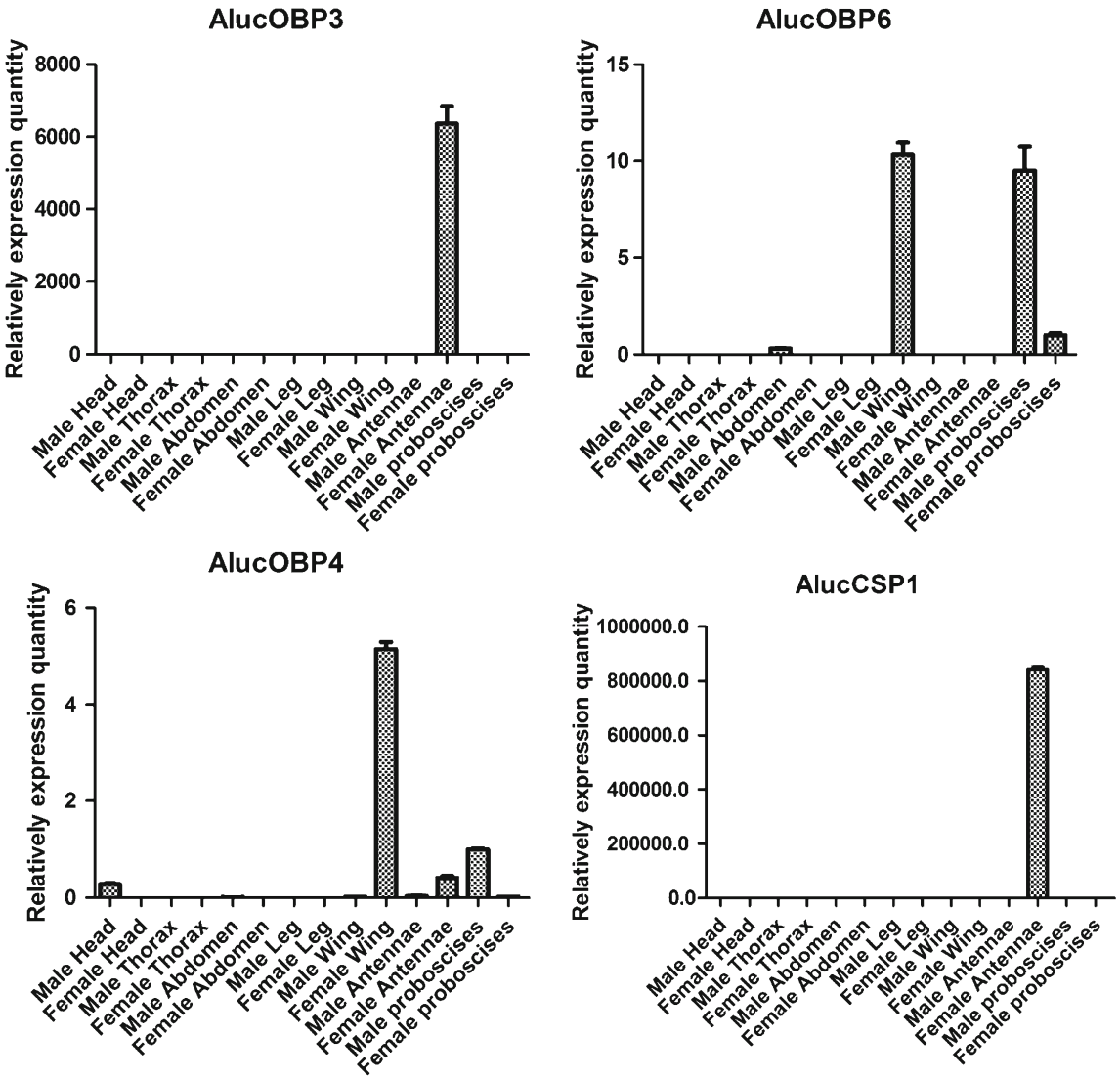

Fig. 3 Relative odorant-binding protein and chemosensory protein transcript levels in different tissues of male and female adult *Apolygus lucorum* evaluated by qPCR. The standard errors of the means of three technical replicates are represented by error bars

over-expression in BL-21 *Escherichia coli* cells as reported previously (Zhang et al., 2011). Specific primers (Table 2) were used to amplify the coding sequence of AlucOBPs and AlucCSP genes with the cDNA as the template. GSTrap FF prepacked columns (GE Healthcare, Piscataway, NJ, USA) were used for purification and thrombin solution (1 U/μl) (GE Healthcare) was used to remove the GST tag. The purified proteins then were desalted using desalting columns. Proteins from the collected fractions resolved by SDS-PAGE and the purified recombinant proteins were stored at −80 °C (Fig. 1).

Fluorescence Experiments Fluorescence measurements were performed as reported previously (Qiao et al., 2009), on an F-4500 fluorescence spectrophotometer (Hitachi, Japan) in a 1 cm light-path fluorimeter quartz cuvette at 25 °C. The fluorescent probe was 4, 4′-dianilino-1, 1′-binaphthyl-5, 5′-sulfonate (bis-ANS, Sigma, Riedstr, Steinheim, Germany) with excitation at 365 nm and emission spectra recorded from 435 to 580 nm.

Dissociation constants of the competitors were calculated from the corresponding IC_{50} values, by using the equation: $Ki = [IC_{50}]/1 + [\text{bis} - \text{ANS}]/K_{\text{bis-ANS}}$, where [bis-ANS] is the free concentration of bis-ANS and $K_{\text{bis-ANS}}$ is the dissociation constant of the protein/bis-ANS complex (Wei et al., 2008).

Results

Library Construction and Database Analysis A cDNA library was constructed from proboscises of *A. lucorum*. The titre of the library was 4.3×10^6 pfu/ml, indicating a sufficient number of genes cloned. Approximately 1800 cDNAs with inserts greater than 0.4 kb were sequenced from the library, resulting in 1554 high quality ESTs for further analysis. The 1554 ESTs from *A. lucorum* were assembled into 658 unigenes, of which 111 were contigs and 547 were singletons (83 %).

Among the 658 unigenes, 111 (17 %) had BLASTX hits at an E-value cut-off of 1.0 E-5 in the non-redundant database, whereas the remaining 547 (83 %) showed no significant similarity to any existing entries in GenBank. Homology searches in the Gene Ontology (GO) database resolved 19 % of the unigenes for roles in cells or cellular components, 24 % in binding and catalytic activity, and 31 % in cellular processing.

We searched for homologs of the 658 unigenes in other insect species using BLASTX program with the E-value cut-off of 1.0 E-5. The top best hits insect species were *Tribolium castaneum* (4.7 %), *Melampsora larici-populina* (2.6 %), *Drosophila melanogaster* (2.4 %), *Acyrthosiphon pisum* (2.4 %), *Aedes aegypti* (2.3 %), *Apis mellifera* (2.3 %),

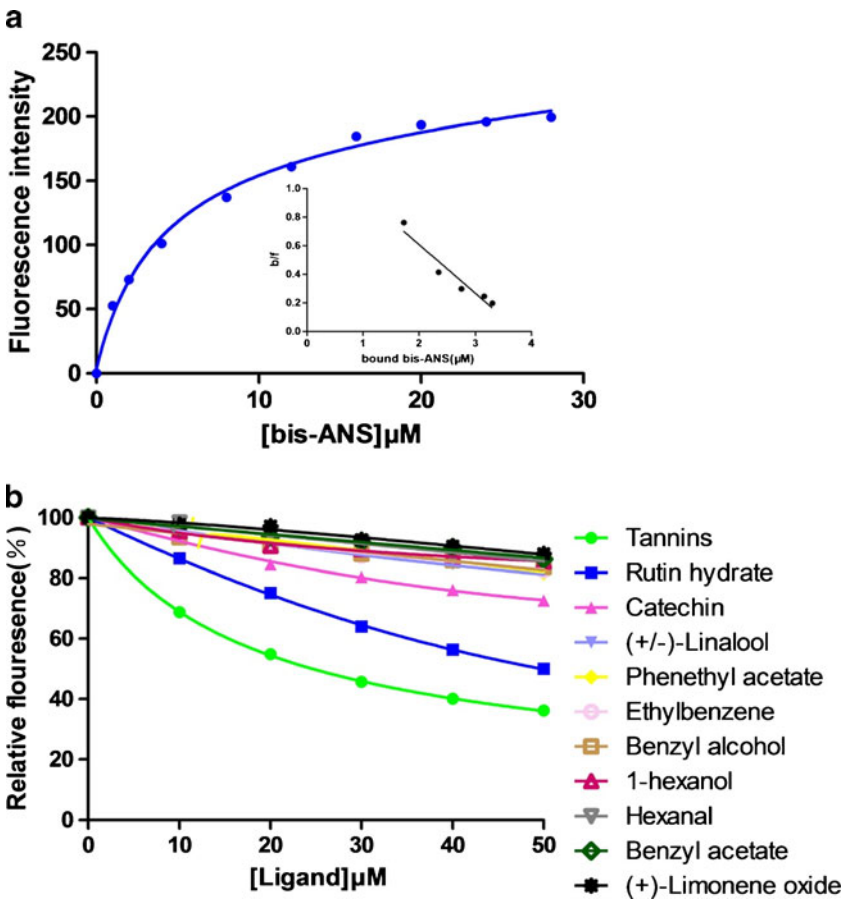

Fig. 4 Binding curve for 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate acid (bis-ANS) to recombinant *Apolygus lucorum* OBP3 (**a**) and Scatchard plot (inset). A 2 μM solution of the protein in Tris buffer was titrated with a 2 μM solution of bis-ANS in methanol to final concentrations of 2–35 μM. Dissociation constants (mean of three replicates) were: AlucOBP3:4.8± 0.5 μM; AlucOBP4: 2.1± 0.4 μM; AlucOBP6:3.4± 0.2 μM; AlucCSP1:4.8±0.3 μM. (**b**) Competitive binding of selected ligands to AlucOBP3. A mixture of the protein (4 μM) and bis-ANS (4 μM) in Tris buffer was titrated with a 4 μM solution of each competing ligand to final concentrations of 2–50 μM. Fluorescence intensities are reported as percentages of the values in the absence of competitors. The same potential ligands were also used with other OBPs and CSP1 (data not shown)

Camponotus floridanus (2.3 %), *Harpegnathos saltator* (2.3 %), *Anopheles gambiae* (2.3 %), with 46.3 % homologous to genes of other insects, but only 1.6 % homologous with genes from *A.lucorum*.

Identification the OBP and CSP Genes The most abundant transcripts (13 %) (207 out of 1554 ESTs) in the library were homologs of OBPs as well as other chemosensory proteins such as chemosensory protein (CSP; 1 %). Three putative OBP genes (AlucOBP3, AlucOBP4, AlucOBP6) and one CSP gene (AlucCSP1) were annotated from the proboscis cDNA library (Table 3). They contain the characteristic sequence features for these proteins, such as conserved cysteine residues (six in the OBPs and four in the CSP) and signal peptides (Fig. 2).

The similarities among the three OBPs are 36 % (AlucOBP3-AlucOBP4), 27 % (AlucOBP3-AlucOBP6) and 24 % (AlucOBP4-AlucOBP6). Through Blast alignment with the GenBank sequences, AlucOBP3 shares the highest similarity with AlinOBP11 (*Adelphocoris lineolatus* odorant-binding proteins 11) (95 %), AlucOBP4 shares the highest similarity with AlinOBP8 (98 %), whereas AlucOBP6 shares the highest similarity with AlinOBP6 (96 %) (Fig. 2).

Tissue-Specific Expression of the OBP and CSP Genes qPCR was used to investigate the presence of AlucOBPs and AlucCSP1 in different tissues of *A. lucorum*. For sample analysis, the comparative $2_T^{-\triangle\triangle C}$ method was used to quantify the results obtained for AlucOBPs and AlucCSP1 transcripts. All samples were normalized using 18S rRNA levels as a reference. The absolute values of the slope of all lines from template dilution plots (log cDNA dilution vs. $\triangle C_T$) were close to zero (data not shown).

All the AlucOBPs and AlucCSP1 have distinct patterns of expression (Fig. 3). AlucOBP3 was expressed in antennae and proboscises of both sexes, and in female wings or male heads. AlucOBP3 was highly expressed in female antennae. AlucOBP4 was expressed in wings, antennae, and proboscises of both sexes, but only in male heads. AlucOBP4 was highly expressed in female antennae, about 5–fold higher than in male proboscises where AlucOBP4 expression was next highest. AlucOBP5 was expressed in proboscises of both sexes, only in male abdomen or male wings. AlucOBP5 was most highly expressed in male wings and proboscises, about 10–fold higher and 9–fold higher than in female proboscises, respectively. AlucCSP1 was expressed in heads, wings, antennae, and proboscises of both sexes, and only in male abdomen. AlucCSP1 was highly expressed in female antennae.

Binding Specificities of AlucOBPs and AlucCSP1 The four purified proteins were used in competitive binding assays with the fluorescent probe bis-ANS as a reporter, and the 63 potential ligands, including 58 volatile compounds and five cotton secondary metabolites, as competitors. First, we measured the dissociation constants between AlucOBPs and bis-ANS. Values ranged from 2.1 μM to 4.8 μM (Fig. 4). Competitive binding experiments revealed different specificities across the four proteins (Table 4). AlucOBP3 bound 8 volatile compounds, whereas AlucOBP4, AlucOBP6, and AlucCSP1 bound 7, 4, 3 volatile compounds, respectively, but all the volatile compounds could not effectively displace

Table 4 Dissociation constants (mean ± SD) (μM) of *Apolygus lucorum* odorant binding proteins and chemosensory protein for potential ligands using the fluorescent probe bis-ANS

Potential ligand[a]	AlucOBP3	AlucOBP4	AlucOBP6	AlucCSP1
Phenethyl alcohol	N.D[b]	>100	N.D	N.D
Benzyl alcohol	>100	N.D	N.D	N.D
Benzyl acetate	>100	N.D	>100	N.D
Phenethyl acetate	>100	N.D	>100	N.D
Ethyl benzene	>100	N.D	N.D	N.D
Hexanal	>100	N.D	N.D	N.D
1-hexanol	>100	N.D	N.D	>100
Cis-3-hexenal	N.D	N.D	N.D	N.D
Cis-3-hexen-1-ol	N.D	>100	N.D	N.D
(+)-Limonene oxide	>100	N.D	N.D	N.D
(+)-Carvone	N.D	N.D	>100	N.D
(+/-)-Linalool	>100	N.D	>100	N.D
Geranyl acetate	N.D	N.D	N.D	>100
Eucalyptol	N.D	N.D	>100	N.D
Indole	N.D	N.D	>100	N.D
Ethyl butyrate	N.D	>100	N.D	N.D
Trans-2-hexenyl butyrate	N.D	>100	N.D	>100
Hexyl butyrate	N.D	>100	N.D	N.D
Heptyl acetate	N.D	>100	N.D	N.D
Methyl salicylate	N.D	>100	N.D	N.D
Gossypol	N.D	N.D	7.5±0.5	13.4±1.4
Tannins	16.1±1.2	12.3±0.3	10.1±0.4	32.7±2.4
Catechin	>100	12.3±0.1	51.5±2.3	>100
Quercetin	N.D	19.7±0.3	17.9±0.9	19.7±0.7
Rutin hydrate	26.2±1.8	16.43±0.9	15.2±1.2	38.5±1.5

[a] More potential ligands were tested, but the remaining 38 potential ligands did not bind any of the OBPs and CSP1. These compounds were Benzaldehyde, Phenylacetaldehyde, Methyl anthranilate, *trans*-2-hexenal, 3-hexanone, 2-hexanone, 3-hexanol, *cis*-2-hexen-1-ol, Geraniol, β-cyclocitra, Damascenone, Geranylacetone, β-caryophyllene, Farnesene, *Trans*-nerolidol, α-phellandrene, (+/−)-α-pinene, Camphene, (+)-β-pinene, (R)-(+)-limonene, Myrcene, Naphthalene, 6-methyl-5-hepten-2-one, Tridecane, Tetradecane, 2-heptanone, 2-hexyl-1-decanol, *cis*-3-hexenyl acetate, α-ionol, β-ionone, Butyl formate, Caproyl acetate, Butyl acetate, Octyl aldehyde, *cis*-3-hexenyl hexanoate, Undecane, Dodecane, Decanal

[b] N.D. is an abbreviation for not detected, which means that no significant binding was detected in the assay

bis-ANS from the AlucOBPs or AlucCSP1. Of the five cotton secondary metabolites tested, AlucOBP3, AlucOBP4, AlucOBP6, and AlucCSP1 bound 3, 4, 5, and 5 compounds, respectively. In comparison with the volatile compounds, the cotton secondary metabolites can effectively replace bis-ANS from the AlucOBPs or AlucCSP1.

Discussion

Insects can detect complex environmental stimuli such as volatile and non-volatile compounds, and respond to them. The binding of OBPs and CSPs with such compounds is the first step for insects to identify odors and tastants that are important for their survival and reproduction (Vogt et al., 1999; Ronderos and Smith, 2009).

To study the molecular mechanisms involved in the gustatory sensory system of *A. lucorum* we constructed a cDNA library from proboscises of *A. lucorum*. The transcripts of OBP and CSP genes are abundant in *A. lucorum* proboscises. The amino acid sequence homology among AlucOBPs was 24–46 %, compared with homology to *Hemiptera* OBPs which ranged from 8 % to 98 %. There is also homology between the AlucOBPs found in proboscises and OBPs from antennae of *A. lineolatus*, indicating that some OBPs may have roles in both olfaction and gustation.

The expression patterns of genes in different tissues and sexes can provide clues to gene function. To investigate the roles of AlucOBPs and AlucCSP1 we used qPCR to determine their expression levels in different *A. lucorum* tissues and sexes. All four genes were expressed mainly in antenna, proboscis, and leg, suggesting roles in olfaction and gustation. Both AlucOBP3 and AlucCSP1 were highly expressed in female antenna, suggesting a major olfactory role of these proteins in female *A. lucorum*. AlucOBP3 and AlucCSP1 also were expressed in proboscises and wings, suggesting that they may also have a gustatory role. AlucOBP4 was highly expressed in female wings and male proboscises, suggesting it too may have a gustatory function. AlucOBP6 was highly expressed in male wings and male proboscises, suggesting it may be involve in gustation in male *A. lucorum.*

Since the AlucOBPs and AluCSP1 may be involved in both olfactory and gustatory function, we explored their ligand binding specificity for plant volatiles and cotton secondary metabolites. In total, 58 compounds were tested, including nine green leaf volatiles, 21 aliphatic alcohols, eight aromatic compounds, 17 terpenoids, and three heterocyclic compounds. Of the 58 compounds examined, 27 were from cotton, healthy or damaged by *H. armigera* (Yu et al., 2010). Five secondary metabolites, including gossypol, tanin, catechin, quercetin, and rutin hydrate also were tested. AlucOBPs and AlucCSP1 can bind plant volatiles, but plant volatiles were not able to effectively displace bis-ANS from the AlucOBPs or AlucCSP1. In comparison, most of the cotton secondary metabolites showed a high affinity with the AlucOBPs and AlucCSP1, indicating that these binding proteins may be involved in both olfactory and gustatory function, but with the main function being gustation. These results will contribute to understanding the role of gustation in detecting food by *A. lucorum* and also will provide a foundation for the development of novel pest control strategies against this cotton pest.

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Potential of Synthetic Sex Pheromone Blend for Mating Disruption of the Swede Midge, *Contarinia nasturtii*

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Abstract The potential for pheromone-based mating disruption of the *Brassica* pest *Contarinia nasturtii* was tested, both in small-scale plots with Brussels sprouts and in commercial-scale fields with either broccoli or cauliflower. Experiments in the small-scale plots used laboratory-reared insects released into a previously uninfested area, whereas large-scale experiments used a high natural population of *C. nasturtii*. Effectiveness of mating disruption was evaluated by the reduction of male captures in pheromone traps, and by reduction of crop damage caused by *C. nasturtii*. Dental cotton rolls (small-scale experiment) and polyethylene caps (large-scale experiment), containing 50 μg (2*S*, 9S)-diacetoxyundecane, 100 μg (2*S*,10*S*)-diacetoxyundecane, and 1 μg (2*S*)-acetoxyundecane, spaced 2 m apart, served as dispensers in the test plots. In both experiments, mean catches of *C. nasturtii* males in pheromone traps were reduced to near zero in treated plots, with control plots averaging 71 males/trap. In the large-scale experiments, no males were caught in pheromone traps over a period of 41 days after mating disruption was applied; one male was caught from days 42–60. In the small-scale trials, crop damage was reduced by 59 %, compared to the untreated control plot. In the large-scale experiments, damage was reduced on average by 91 %. This study shows successful field application of the mating disruption technique for control of a member of the dipteran family Cecidomyiidae, and demonstrates that pheromone-based mating disruption has potential for management of *C. nasturtii* populations.

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Introduction

The Cecidomyiidae, gall midges, are a dipteran family containing about 5000 species (Gagné, 1989), of which a number are important agricultural pests. The swede midge, *Contarinia nasturtii* (Kieffer), is a serious pest infesting Brassicaceae, e.g., broccoli, cauliflower, cabbage, and Brussels sprouts. Even relatively low population levels of this midge can cause high levels of damage (Harris, 1966; Darvas et al., 2000). The midge is widespread and economically important in Europe, and was introduced into Canada in the mid-1990s (Hallett and Heal, 2001), and later into the USA (Kikkert et al., 2006). The initial infestations in Ontario, Canada caused up to 85 % crop losses on some farms (Hallett and Heal, 2001). *Contarinia nasturtii* has spread to several important vegetable and canola growing regions in North America (Chen et al., 2011).

A pheromone-based monitoring system has been developed for adult males of *C. nasturtii*, using a blend of (2*S*,9*S*)-diacetoxyundecane, (2*S*,10*S*)-diacetoxyundecane, and (*S*)-2-acetoxyundecane (Hillbur et al., 2005; Boddum et al., 2009). Effective control of this pest requires a number of insecticide applications at weekly or even shorter intervals (Baur et al., 2005; Chen et al., 2007, 2011). Sustainable, cost-effective methods need to be developed to control the pest at low and high infestations.

Female *C. nasturtii* lay eggs in clusters on young plant tissue (Barnes, 1946; Readshaw 1961). The larvae feed at the growing tips of a plant, leading to gall-like distortions,

turning growing tips, deformed plant tissue, and corky brown scars. In Brussels sprouts, cauliflower, broccoli, and other *Brassica* vegetable crops, even the slightest damage leaves the yield unmarketable. When larvae are fully developed, they move into the soil, form cocoons, and either pupate, before emerging to start the next generation cycle, or begin a diapause that can be one or two winters long. Depending on the regional climate, *C. nasturtii* has three to four generations per year (Readshaw 1961; Hallett et al., 2009b). In spring, diapausing larvae leave the winter cocoon and create a summer cocoon in which they pupate and then eclose. Emergence is determined by temperature and moisture (Readshaw, 1966).

Common characteristics for cecidomyiid midges are their small size (a few mm) and the short life span of adults (1–2 days) (reviewed by Hall et al., 2012). In many species, males mate several times, whereas females mate only once and produce monogenous progeny (*ibid.*). Given that *C. nasturtii* mate shortly after emergence (Barnes, 1946) and that the flight range of adults is relatively short (Samietz et al., unpublished data), there may be potential to control damage by this midge through use of pheromone mating disruption. Accordingly, the aim of this study was to trial an effective and environmentally acceptable control of *C. nasturtii* by applying synthetic sex pheromone of *C. nasturtii* to prevent mating, and consequently reproduction, of this pest in crops.

Methods and Materials

Pheromone Dose and Dispensers Dispenser spacing was set to 2×2 m, providing a radius of about 1 m around each dispenser, in order to match the low flight ability of the midges (Samietz et al., unpublished data). Each dispenser was loaded with a blend of 50 μg (2*S*, 9S)-diacetoxyundecane, 100 μg (2*S*,10*S*)-diacetoxyundecane, and 1 μg (2*S*)-acetoxyundecane, diluted in 100 μl of hexane, resulting in a dose 100 times higher than the dose used for monitoring (Hillbur et al., 2005). Freshly loaded dispensers were placed in the crop 3 d prior to experiments.

The pheromone used in the small-scale experiments was loaded in dispensers made of whole dental cotton rolls (14 mm diam., length 40 mm; IVF Hartmann, Neuhausen, Switzerland). Each dispenser was covered by a delta-shaped, 10 cm-wide sheet (10 cm high; open to the sides), made of white plastic foil, for rain protection, and mounted at a height of 45 cm.

In the large-scale experiments, polyethylene vial caps (12 mm diam., height 8.5 mm, Semadeni, Ostermundigen, Switzerland) were used, as polyethylene is a longer-lasting dispenser than cotton in the field (Boddum et al., 2009). The vial caps were pressed through holes, matching their diameter, in white plastic cross-slit labels (Kreuzschnittetiketten 4×8 cm; Baumann Saatzuchtbedarf, Waldenburg, Germany). One dispenser was adjusted to one plastic cross-slit stick of 40 cm length (Kreuzschnittetikettenstäbe; Baumann Saatzuchtbedarf, Waldenburg, Germany). Another cross-slit label (c.f. above), without a hole, was slipped on the same stick exactly above the dispenser, in order to protect it from rain and sunlight (especially ultraviolet radiation). The ready-made sticks were fixed about 15 cm deep into the soil; i.e., dispensers were at a height of about 25 cm above ground. Again, dispensers were spaced in a rectangular 2×2 m grid over the entire plot.

The field doses of pheromones were estimated based on approximate release rates from trapping dispensers in the field (S. Rauscher, unpublished data). Additional field experiments showed that the release-rate ratio of the three components was relatively constant over a period of 32 d in the field (Boddum et al., 2009). Polyethylene dispensers were highly attractive in the field for a period of 46 d, despite comparatively low release rates toward the end of the period (Boddum et al., 2009). In dental cotton dispensers, the amounts of pheromone components released during the field trials over 70 d were estimated to average 1.78 $mg \cdot ha^{-1} \cdot day^{-1}$ (2*S*, 9S)-diacetoxyundecane, 3.57 $mg \cdot ha^{-1} \cdot day^{-1}$ (2*S*,10*S*)-diacetoxyundecane, and 35.7 $\mu g \cdot ha^{-1} \cdot day^{-1}$ (2*S*)-acetoxyundecane. In the polyethylene dispensers, the amounts released over 100 d were estimated to average 1.25 $mg \cdot ha^{-1} \cdot day^{-1}$ (2*S*, 9S)-diacetoxyundecane, 2.5 $mg \cdot ha^{-1} \cdot day^{-1}$ (2*S*, 10*S*)-diacetoxyundecane, and 25 $\mu g \cdot ha^{-1} \cdot day^{-1}$ (2*S*)-acetoxyundecane.

Small-Scale Experiments Two equally designed and planted plots of Brussels sprouts (*Brassica oleracea* var. *gemmifera* cv. Helemus) were used with released midges. The plots were located on the ACW experimental farm at Wädenswil (Switzerland). Dimensions of the plots were 16×16 m, with a distance of 1 m between rows, and about 40 plants per row (640 plants per plot). Neither of the plots had *Brassica* planted for several years prior and, as a consequence, had no natural population of *C. nasturtii*. The two plots were located about 500 m apart and were separated by a row of greenhouses, which probably precluded midge movements from one plot to the other and pheromone contamination of the untreated plot. One plot was treated with pheromone for mating disruption; the other was untreated and used as a control.

Contarinia nasturtii were released into plots by placing 12 pots in each plot. Each pot contained substrate with 150–250 pupae of laboratory-reared midges. The total expected emergence of adult midges was about 1000 males and 1000 females per plot per release, hatching over a period of approximately 1 week.

In the initial experiment, starting 20 July 2004, pots were placed in a row 30 m west of the treated and control plots. Plants in the plots, planted 10 weeks before, were about

50 cm high. This experiment tested whether mating disruption in a target field was effective when the emergence area was not treated with pheromone.

In a second experiment, starting 9 August 2004, pots were randomly distributed within the treated and control plots. Plants in plots were planted 13 weeks earlier, and were about 65 cm high. The experiment tested whether mating disruption in a target field is effective when midges emerge within the treated plot. The general effectiveness of mating disruption was tested by pheromone-trap shutdown. For this, four pheromone traps were randomly distributed over each of the treated and control plots. The sticky inserts of the traps were checked, changed, and catches determined at 3- or 4-d intervals over 6 weeks.

Damage for both experiments was scored at harvest time (end of September 2004). Randomly starting within each row, every 5th plant was inspected for damage; i.e., about 130 plants were examined from each of the control and treated plots. Each plant was scored for early damage by 1st-release midges (leaf axillary sprouts on lower plant half) and later damage by 2nd-release midges (leaf axillary sprouts on higher plant half, not yet formed at time of 1st release of midges). Damages scored for each plant were the number of distorted sprouts and the number of sprouts with cork damage.

Large-Scale Experiments Large-scale field experiments were carried out in commercial *Brassica* fields in the Seeland area between Müntschemier and Kerzers in Switzerland. This area is one of the most important Swiss vegetable growing areas, with many medium-size vegetable fields. The breeding conditions for *C. nasturtii* are good in this area, due to the bog soil and moist conditions supported by irrigation, with the population densities being high.

The first experiment was conducted in a 3000-m^2 broccoli field (*Brassica oleracea* var. *italica* cv. Fiesta) in Müntschemier, in two plots planted 29 July 2005 (plot 1.1, 100×15 m) and 12 July 2005 (plot 1.2, 100×15 m), respectively. The treated area within plots was 34×34 m (1156 m^2), using 16×16 (256) pheromone dispensers. The treated plot produced 504 plants at harvest, while the control plot, without any treatment, was 16×30 m (480 m^2) and produced 980 plants at harvest.

The second experiment was conducted in a 8000-m^2 broccoli field (*Brassica oleracea* var. *italica* cv. Fiesta) in Kerzers, planted 1 August 2005 (plot 2). The treated area was 34×42 m (1428 m^2), and used 16×20 (320) pheromone dispensers. It produced 635 plants at harvest. The control area, without any treatment, was 16×40 m (640 m^2) and produced 677 plants at harvest.

The third experiment was conducted in a 5250-m^2 cauliflower field (*Brassica oleracea* var. *botrytis* cv. Fremont) in Ried, planted 2 August 2005 (plot 3). The treated area was 34×42 m (1428 m^2), used 16×20 (320) pheromone dispensers, and produced 338 plants at harvest. The control area, without any treatment, was 10×35 m (350 m^2), and produced 346 plants at harvest.

All pheromone dispensers were placed in the fields on 2 August 2005. Damage scoring was carried out on 30 September 2005 (the two broccoli fields, plots 1.1, 1.2, and 2) and on 18 October 2005 (the cauliflower field, plot 3), scoring each plant (sample sizes given above) in the treated and control plots into the following categories: A—no damage, B—cork damage, C—deformed inflorescence, D—without inflorescence. Only category A would be marketable in Switzerland.

Statistics Pheromone-trapping results were compared using repeated measures analyses of variance (RM-ANOVA), with sampling times being the repetitions in the analysis, followed by a pairwise comparison of treated and control plots by Dunn-Sidak tests. Damage in the small-scale experiments

Table 1 Catches of *Contarinia nasturii* males in pheromone traps (cumulative and per interval) in Brussels sprouts plots where *C. nasturtii* males and females were released within the plots. Numbers in the column marked "Pheromone" refer to trap catches in the plot where pheromone dispensers for mating disruption were applied; the other "Control" plot was left untreated. N=4/plot

Dispenser	Cumulative catches		Catches per trap (mean)				Dunn-Sidak tests	Cumulative ratio
Exposure (d)	Pheromone	Control	Pheromone	SE	Control	SE	Pheromone vs. Control	Control/total catches
7	0	183	0.0	0.0	46	3.8	$P<0.001$	100 %
11	0	247	0.0	0.0	16	1.5	$P<0.001$	100 %
14	0	262	0.0	0.0	3.8	1.1	$P=0.05$	100 %
18	0	271	0.0	0.0	2.2	1.1	$P<0.05$	100 %
21	0	275	0.0	0.0	1.0	0.4	$P<0.05$	100 %
25	0	281	0.0	0.0	1.5	0.6	n.s.	100 %
28	0	282	0.0	0.0	0.2	0.2	n.s.	100 %
32	1	283	0.2	0.2	0.2	0.2	n.s.	99.6 %
35	1	283	0.0	0.0	0.0	0.0	–	99.6 %

was analyzed by analyses of covariance (ANCOVA), with treatment as factor and spatial arrangement (crop row from immigration side) as covariate. Proportions of plants in the different damage categories of the field-scale trials were compared by χ^2 tests for analyzing the tabulated counts. Efficacy of pest control in each trial was calculated as relative reduction of damage in the treated vs. the control plot (Abbot's formula). All statistics were performed with XLStat Pro V2011.204 (Addinsoft, Andernach, Germany).

Results

Small-Scale Experiments Application of pheromone resulted in a complete reduction of trap catch in pheromone traps in the plot where midges were released inside the plot (Table 1); no midges were caught over four continuous weeks (RM-ANOVA, treatment by repetition: $F_{7,42}=100.3$, $P<0.001$). During the same period, traps in control plots caught more (282) midges (Table 1; Dunn-Sidak tests, $P<0.05$). Five weeks after release, a single male was caught in the treated plot (Table 1).

In total, 79.4 % of all plants in controls of the small-scale trials were damaged by *C. nasturtii*, indicating that the release of midges simulated high population pressure. The spatial distribution of damage in the control and pheromone-treated plots is shown in Fig. 1. The total damage across the sampled plants (Fig. 1a) was influenced by treatment (ANCOVA factor: $F_{1,260}=37.5$, $P<0.001$), but not by spatial arrangements of rows from the initial immigration side, i.e., in the first experiment with release from distance (ANCOVA covariate: $F_{1,260}=0.227$, $P=0.634$). The same result was obtained when analyzing separately for early damage caused by midges released from distance (Fig. 1b; ANCOVA factor: $F_{1,260}=6.62$, $P=0.011$, covariate: $F_{1,260}=0.001$, $P=0.973$), and also for late damage caused by midges released from inside the plots (Fig. 1 c; ANCOVA factor: $F_{1,260}=51.3$, $P<0.001$, covariate: $F_{1,260}=0.459$, $P=0.499$).

The pheromone treatment resulted in a reduction of damage by *C. nasturtii*, both when midges were released at a distance from the plots, and also when they were released inside plots (Table 2). When midges were released from a distance, crop damage was reduced by 68 % (ANCOVA factor: $F_{1,260}=4.00$, $P<0.05$, covariate: $F_{1,260}=0.237$, $P=0.627$), when measured by the number of distorted sprouts, and by 80 % (ANCOVA factor: $F_{1,260}=5.22$, $P<0.05$, covariate: $F_{1,260}=0.002$, $P=0.962$), when measured by cork-damaged sprouts. Damage was also reduced when midges were released within the field; by 75 %, when measured by the number of distorted sprouts (ANCOVA factor: $F_{1,260}=49.2$, $P<0.001$, covariate: $F_{1,260}=1.39$, $P=0.238$), and by 34 %, when measured by the cork-damaged sprouts (ANCOVA factor: $F_{1,260}=11.7$, $P<0.001$, covariate: $F_{1,260}=2.99$, $P=0.082$). The overall reduction of damage in the two release experiments was 59 % (Table 2; ANCOVA factor: $F_{1,260}=37.5$, $P<0.001$).

Large-Scale Experiments In the commercial fields, no *C. nasturtii* males were caught in pheromone traps over the first 41 days after the mating disruption treatment had been applied; from days 42–60, one male was caught (Fig. 2; Dunn-Sidak tests, $P<0.05$). In the untreated control, 473 males were caught during the first 41 days, and 239 males caught from days 42–60. Over the entire period, the

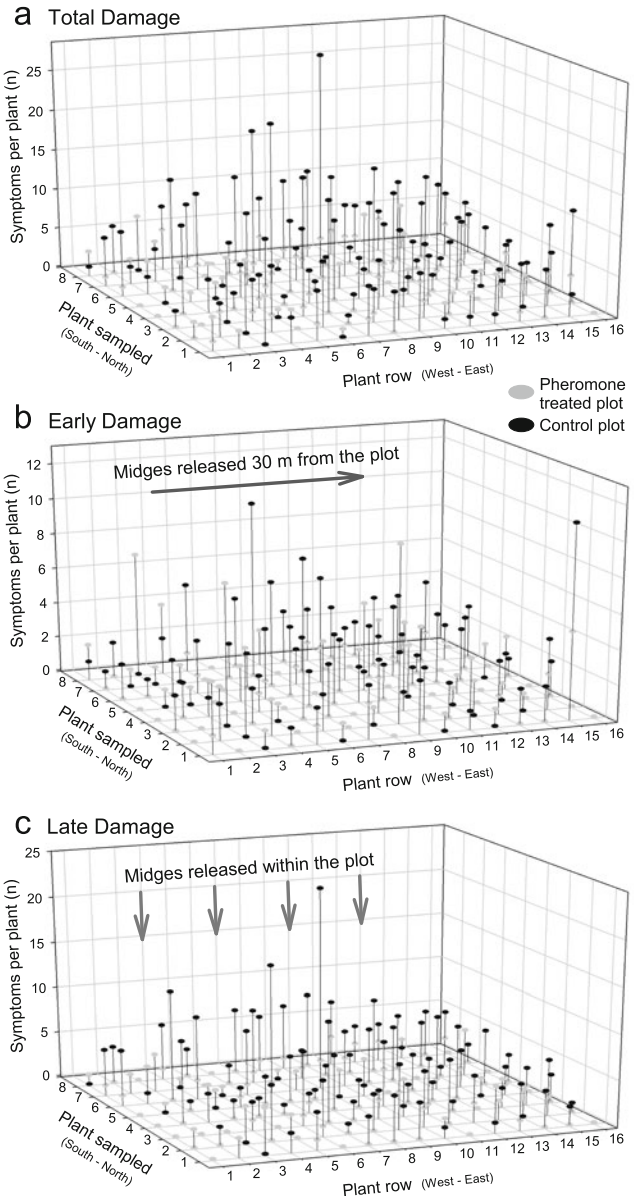

Fig. 1 Spatial distribution of *Contarinia nasturtii* damage of individual Brussel sprout plants in small-scale mating disruption trials. Number of damage symptoms per sampled plant **a** in total, **b** with release of midges 30 m from the plot, and **c** with release of midges within the plot. See text for statistics

Table 2 Damage caused by *Contarinia nasturtii* in Brussels sprouts in a plot with pheromone dispensers for mating disruption (Pheromone) vs. an untreated (Control) plot

Damage type related to *C. nasturtii*	Release of midges causing damage	Number of sprouts damaged per plant				Treatment efficacy
		Pheromone		Control		
		Mean	SE	Mean	SE	
Distorted sprouts (A) (lower plant half)	Midges released 30 m from plot	0.95	0.12	3.00	0.27	68 %
Cork-damaged sprouts (B) (lower plant half)	As above	0.06	0.02	0.31	0.07	80 %
Distorted sprouts (C) (higher plant half)	Midges released within plot	0.02	0.02	0.09	0.03	75 %
Cork-damaged sprouts (D) (higher plant half)	As above	1.00	0.16	1.52	0.17	34 %
Sum of symptoms (A–D)		2.03	0.21	4.92	0.42	59 %
Sample size (Number of plants)		130 (20 % of all plants sampled from each treatment)		131		

difference in trap catches between treated and control plots was highly significant (RM-ANOVA, treatment by repetition: $F_{8,48}=5.27$, $P<0.001$).

Untreated control plots of the field trials had rates of damaged plants between 7.4–19.1 % (average 12.4±2.5 %, SE). All three fields included in the large-scale experiment showed a reduction in damage by *C. nasturtii* (Fig. 3, χ^2-tests, $P<0.01$). Total damage rates in the untreated control ranged from 10–19 % (Fig. 2a). The reduction in crop damage due to pheromone treatment was 90.9 %, on average (Fig. 3a). When discriminating for damage type, cork damage (corky scars on plant tissue), deformed inflorescence, and missing inflorescence, the different plots had similar patterns, except for the 1st planting in Müntschemier, in which plants in the pheromone-treated areas showed no deformed or missing inflorescences, implying a 100 % mating disruption efficacy for these damage types (Fig. 3b–d).

Discussion

Our study showed that the tested release rates of (2*S*,9S)-diacetoxyundecane, (2*S*,10*S*)-diacetoxyundecane and (2*S*)-acetoxyundecane prevented male capture of *C. nasturtii* in pheromone traps, and also reduced plant damage caused by this pest. This demonstrates the potential of pheromone-based mating disruption for control of these cecidomyiid midges, supporting previous pheromone-based control studies, using attract and kill, mass trapping, and mating disruption techniques, on apple leaf curling midge, *Dasineura mali*, and raspberry cane midge, *Resseliella theobaldi*, (reviewed by Hall et al., 2012),

Polyethylene dispensers generated complete trap shutdown over a period of 41 days, and close to 100 % reduction over 60 days in plots containing released *C. nasturtii*. In plots with natural infestations of *C. nasturtii*, treatment reduced damage by 91 % (cauliflower), 94 %, and 88 % (broccoli) relative to untreated controls. A reduction of damage in treated plots was also achieved when midges were released at a distance from the plots, suggesting that

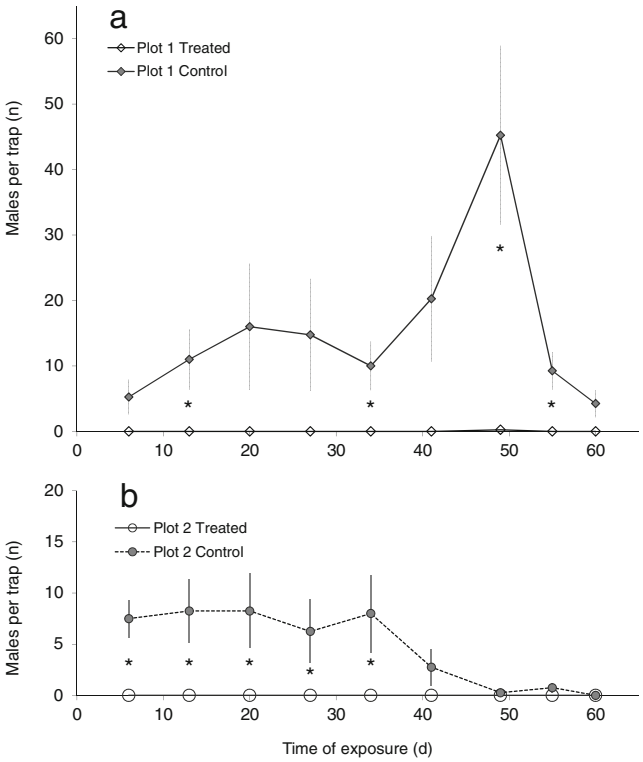

Fig. 2 Mean number (± SE) of *Contarinia nasturtii* males caught in pheromone traps in two large-scale fields with pheromone dispensers for mating disruption, and in two control fields (**a** plot 1, **b** plot 2). In each field plot, four pheromone traps were placed randomly. *Asterisks* indicate differences (RM-ANOVA, Dunn-Sidak tests, $P<0.05$) in trap catches between pheromone treated plots and untreated control plots

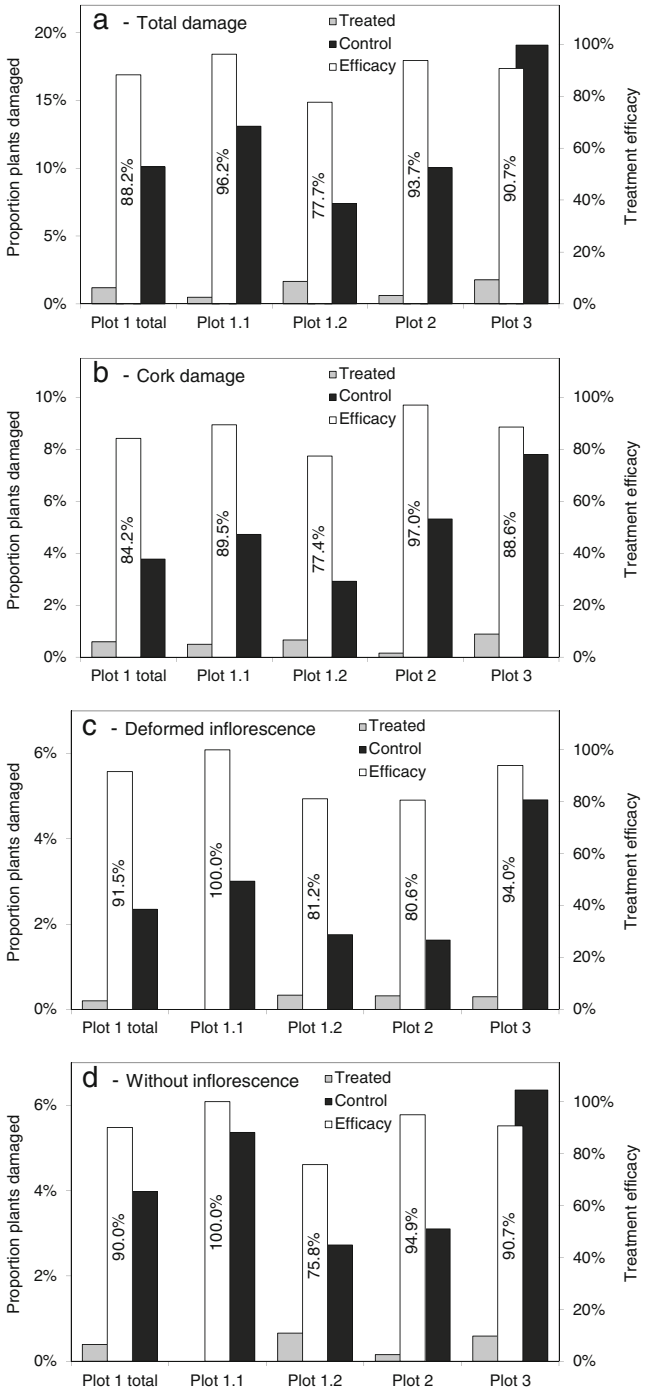

Fig. 3 Damage rates and treatment efficacies in large-scale fields with pheromone dispensers for mating disruption of *Contarinia nasturtii* and in untreated control fields. Proportion of damaged *Brassica* plants in total (**a**) and in the different damage categories (**b**–**d**). All damage categories as well as total damage show differences (X^2-tests, $P<0.01$) between pheromone-treated plots and untreated control plots

a substantial proportion of matings must have occurred away from the site of emergence/release (i.e., probably within control plots). This is promising with respect to the potential of using mating disruption, as the technique may be effective against midges moving into a crop for mating and subsequent oviposition. This also suggests that males, as well as females, may be attracted to the *Brassica* host plant, in contrast to what has been suggested for the related pea midge, *C. pisi* (Wall et al., 1991).

Pyrethroid, spinosyd, and neonicotinoid insecticides are efficient against *C. nasturtii* in the laboratory and in the field, as long as population densities are moderate (Wu et al., 2006; Chen et al., 2007; Hallett et al., 2009a). Damage levels in *Brassica* vegetable cultures are kept within the range of marketable quality at the beginning of the season by regular application of these insecticides (Wu et al., 2006; Hallett et al., 2009a). Under high population densities, later in season, however, the efficacy of insecticides is much lower than what we achieved by pheromone mating disruption in the present study (Hallett et al., 2009a). Our large-scale trials, comparable to high population pressures under natural field conditions, showed a 90 % reduction in damaged plants compared to control plots in which nearly 20 % of the plants were damaged (plot 3).

However, mating disruption is likely to be relatively expensive, due to the relatively high costs of synthetic pheromone. The three components tested were all pure *S*-enantiomers, for which the syntheses are laborious and time-consuming (Hillbur et al., 2005). A possible cheaper option would be to use mixtures of stereoisomers of the three components, with total amounts increased to account for the lower amounts of the correct enantiomers in the racemates. The presence of *R*-enantiomers of two (2,9-diacetoxyundecane and 2-acetoxyundecane) of the three components in the attractive three component blend of *S*-enantiomers has little or no effect on male orientation and pheromone trap catch in the field (Boddum et al., 2009). We would expect, at least for these two components, that mating disruption with racemates should also work by interfering with mate location. In contrast, the presence of the non-natural stereoisomers of 2,10-diacetoxyundecane, i.e., (2*R*,10*R*)-diacetoxyundecane or *meso*-2,10-diacetoxyundecane, has a strong inhibitory effect on male orientation and trap catch (Boddum et al., 2009). Nevertheless mating disruption could be effective with a cheap mixture of stereoisomers, based on a combination of disruptive and repellent effects (Bengtsson et al., 1994; Suckling and Burnip, 1996; Eizaguirre et al., 2007).

Although the influence of dispenser spacing and active dose range remains to be investigated, the synthetic pheromone blend tested in this study shows potential for managing *C. nasturtii* populations. Its use may substitute for, or at least reduce the input of, chemical insecticides (Wu et al., 2006; Hallett et al., 2009a), or enhance other control approaches for this pest, such as cultural techniques or host plant resistance (Wyss and Daniel, 2004; Chen and Shelton, 2007; Hallett, 2007; Chen et al., 2009).

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Mechanisms for Eliminating Monoterpenes of Sagebrush by Specialist and Generalist Rabbits

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Abstract Pygmy rabbits (*Brachylagus idahoensis*) are one of only three vertebrates that subsist virtually exclusively on sagebrush (*Artemisia* spp.), which contains high levels of monoterpenes that can be toxic. We examined the mechanisms used by specialist pygmy rabbits to eliminate 1,8-cineole, a monoterpene of sagebrush, and compared them with those of cottontail rabbits (*Sylvilagus nuttalli*), a generalist herbivore. Rabbits were offered food pellets with increasing concentrations of cineole, and we measured voluntary intake and excretion of cineole metabolites in feces and urine. We expected pygmy rabbits to consume more, but excrete cineole more rapidly by using less-energetically expensive methods of detoxification than cottontails. Pygmy rabbits consumed 3–5 times more cineole than cottontails relative to their metabolic body mass, and excreted up to 2 times more cineole metabolites in their urine than did cottontails. Urinary metabolites excreted by pygmy rabbits were 20 % more highly-oxidized and 6 times less-conjugated than those of cottontails. Twenty percent of all cineole metabolites recovered from pygmy rabbits were in feces, whereas cottontails did not excrete fecal metabolites. When compared to other mammals that consume cineole, pygmy rabbits voluntarily consumed more, and excreted more cineole metabolites in feces, but they excreted less oxidized and more conjugated cineole metabolites in urine. Pygmy rabbits seem to have a greater capacity to minimize systemic exposure to cineole than do cottontails, and other cineole-consumers, by minimizing absorption and maximizing detoxification of ingested cineole. However, mechanisms that lower systemic exposure to cineole may come with a higher energetic cost in pygmy rabbits than in other mammalian herbivores.

Keywords *Artemisia tridentata* · *Brachylagus idahoensis* · Cineole · Detoxification · Pygmy rabbits · *Sylvilagus nuttalli*

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Introduction

Big sagebrush (*Artemisia tridentata*) rangelands dominate 1.1 million km^2 of the western United States and Canada (Meyer and Karasov, 1991), and provide important habitat for a wide range of endemic wildlife species. However, reduction, fragmentation and degradation of sagebrush rangelands has threatened many sagebrush-steppe obligates such as such as sage grouse (*Centrocercus* spp., Connelly et al., 2000), Brewer's sparrows (*Spizella breweri*), sage sparrows (*Amphispiza belli*), sage thrashers (*Oreoscoptes montanus*, Knick and Rotenberry, 2000), Washington, Idaho, and Columbian ground squirrels (*Spermophilus washingtoni, S. brunneus, S. columbianus*, Betts, 1990; Weddell, 1991; Sherman and Runge, 2002), and pygmy rabbits (*Brachylagus idahoensis*, Federal Register, 2003). Despite its importance as wildlife habitat, however, few vertebrate species consume substantial amounts of sagebrush. Only three species, pronghorn (Ngugi et al., 1992), sage grouse (Connelly et al., 2000), and pygmy rabbits

(Thines et al., 2004) consume sagebrush as more than a minor component of their seasonal diets, and thus are considered dietary specialists (Shipley et al., 2009). For example, pygmy rabbits consume up to 99 % of their diet from big sagebrush (*Artemisia tridentata*) in winter and up to 50 % in summer (Green and Flinders, 1980; Thines et al., 2004). Sagebrush contains high concentrations of plant secondary metabolites (PSMs) such as terpenes, sesquiterpene lactones, and phenolics, which can influence intake (Kelsey et al., 1973; Bray et al., 1991; Wilt et al., 1992). Monoterpenes, which comprise 1–4 % of dry mass (DM) of sagebrush (White et al., 1982), generally are toxic to mammals (Nagy et al., 1964; Cork and Foley, 1991), compromise energy budgets (Sorensen et al., 2005), and are avoided by most herbivores (Sinclair et al., 1988; Meyer and Karasov, 1991). Consuming moderate amounts of sagebrush (15–50 % of the diet) have caused digestive upset, reduced rumen motility, rumen lesions, rumenitis, and even death in mice and wild and domestic ruminants (Nagy et al., 1964; Johnson et al., 1976).

Herbivores that regularly consume plants with PSMs like those in sagebrush have developed the ability to avoid or tolerate PSMs through detoxification and elimination. Herbivores have a variety of options when confronted with PSMs in their diet (Fig. 1). First, animals can avoid plants or plant parts containing high amounts of PSMs through conditioned flavor aversion (Provenza, 1995). Second, animals may consume the plant, but avoid absorbing PSMs by releasing volatiles while chewing (White et al., 1982) or by excreting unmetabolized parent PSMs or metabolites in feces. PSMs that are lipid-soluble, nonpolar, and nonionic may be actively effluxed from enterocytes back into the lumen via ATP-dependent transporters and, therefore, excreted unmetabolized in the feces (Sorensen and Dearing, 2006; Stanley et al., 2009). In addition, PSMs can be metabolized by detoxification enzymes in enterocytes (Sorensen and Dearing, 2006; Sorensen et al., 2007) or by microflora (Malecky and Broudiscou, 2009) before absorption and excreted in feces as metabolites.

If absorbed, PSMs are distributed to organs via the circulation where they can accumulate in tissues or be detoxified by metabolizing enzymes (primarily in the liver) and excreted via several routes (Fig. 1). Detoxification of PSMs generally increases water solubility for excretion in urine (McLean and Duncan, 2006; Sorensen et al., 2006), bile (Grigoleit and Grigoleit, 2005), or respired air (Boyle et al., 2002). In Phase I detoxification, microsomal enzymes such as Cytochrome P450 catalyze reactions such as oxidation, reduction, or hydrolysis. The more oxidized the PSM metabolite, the more polar it becomes. In Phase II, PSMs or Phase I metabolites are conjugated to polar molecules, such as glycine and glucuronic acid.

Herbivores, like pygmy rabbits, that eat specialized diets consisting of large quantities of a few taxa of plants containing PSMs must limit the absorption of PSMs and rapidly and efficiently detoxify and eliminate them. Although specialists voluntarily consume more PSMs in their diets than do generalist herbivores, they often employ more effective mechanisms to limit absorption than do their generalist

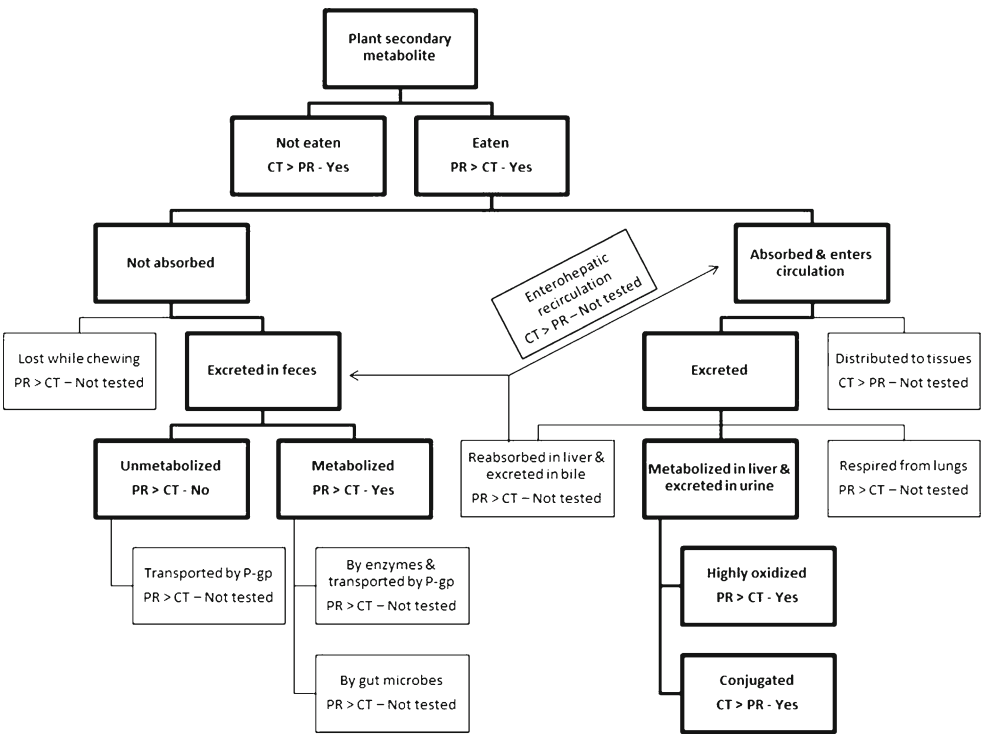

Fig. 1 Possible fates of plant secondary metabolites (PSMs) encountered by herbivores (adapted from Marsh et al., 2006), expected differences in strategies used by specialist pygmy rabbits (PR, *Brachylagus idahoensis*) and generalist cottontails (CT, *Sylvilagus nuttalli*) consuming monoterpenes in sagebrush, and outcome of tests of our hypotheses (in bold) and those not yet tested

counterparts (Sorensen and Dearing, 2003; Sorensen et al., 2004). Furthermore, specialists may use detoxification pathways that are less energetically expensive than do generalists. For example, specialists may oxidize PSMs to a greater degree, thus reducing the need for subsequent conjugation with glucuronic acid, a derivative of endogenous glucose, or with glycine, an amino acid (Boyle et al., 1999, 2000). Finally, specialists (e.g., ringtail possums, *Pseudocheirus peregrinus*, and koalas, *Phascolarctos cinereus*) produce fewer metabolites conjugated with glucuronic acid than do generalists (e.g., lab rats and brushtail possums, *Trichosurus vulpecula*, Boyle et al., 1999, 2000) and excrete less glucuronic acid per unit of energy intake (e.g., Stephens woodrat, *Neotoma stephensi*, Sorensen et al., 2005).

Our goal in this study was to examine mechanisms that allow pygmy rabbits to tolerate nearly monospecific diets of sagebrush by comparing their intake and fecal and urinary excretion of 1,8-cineole, a common monoterpene of sagebrush (Fig. 2, Shipley et al., 2006) and its metabolites, with that of mountain cottontails (*Sylvilagus nuttalli*). Cottontails are generalist herbivores that often co-inhabit sagebrush rangelands with pygmy rabbits in the northwestern United States, but consume diets of <4 % sagebrush year round (e.g., Johnson and Hansen, 1979; MacCracken and Hansen, 1984). We predicted that pygmy rabbits would (Fig. 1): 1) voluntarily consume more cineole than cottontails; 2) excrete more unmetabolized cineole and cineole metabolites in their feces than cottontails; 3) excrete more highly-oxidized cineole metabolites in their urine and feces than cottontails; 4) excrete a lower proportion of conjugated metabolites, thus their urine would have lower amounts of glucuronic acid and gross energy, and a higher pH than cottontails.

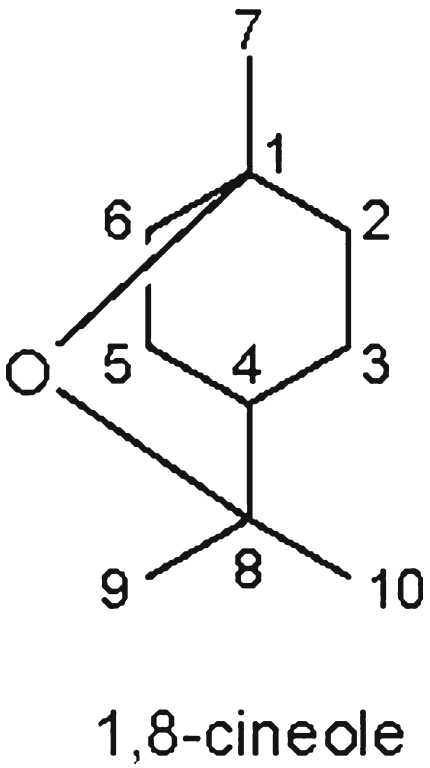

Fig. 2 The common naming and numbering system used throughout this paper for 1,8-cineole and its oxygenated metabolites was based on the *p*-menthane scaffold as shown. Thus, the oxygenated derivatives discussed in the text occur at C2, C3, C7 or C9 and can occur as simple hydroxylations, carboxylations or combinations thereof

Methods and Materials

Measuring Intake of Cineole To compare the tolerance for 1,8 cineole between pygmy rabbits and cottontails, we conducted a series of intake trials with rabbits captured in wire box traps from southern Idaho (pygmy rabbits) and eastern Washington (cottontails). Four pygmy rabbits (3 female, 1 male) weighing a mean of 432 g (SD=83 g) and 6 cottontail rabbits (4 female, 2 male) weighing a mean of 1,174 g (SD= 215 g) were acclimated to a completely-balanced grain-alfalfa rabbit pellet in 0.4-m^2 stainless steel metabolic crates, with fresh water *ad libitum*. All protocols were approved by WSU's Institutional Animal Care and Use Committee (protocol #3097). During trials, rabbits received increasing amounts of 1,8-cineole (99 %, Alfa Aesar, Ward Hill, MA, USA) mixed with pellets in experimental blocks of 3 d per concentration, including 0, 1, 3, 5, and 7 % cineole by dry weight. Concentrations of cineole were achieved by mixing pellets with cineole in an amber-colored glass jar each morning, and storing them in the freezer when not in use. Pellets were weighed before offering them to the rabbits in ceramic bowls, and refreshed 3 times per day to avoid excessive evaporation of cineole from the pellets. Samples of food offered and refused were collected at each feeding for later chemical analysis. We measured the dry matter of food offered by weighing a fresh subsample, drying it at 100 °C for 24 hr, then re-weighing it. We collected all refusals and dried them at 100 °C for 24 hr. On the third day at each cineole concentration, we collected urine from the rabbits on dry ice for 24 hr and froze it at −20 ° C. Although ice was routinely used for urine collection, it was not considered essential because cineole metabolites are stable at room temperature (S. McLean, pers. comm.), and ice was omitted for the feces collection because of practical difficulties. We determined dry matter from a subset of feces. Volume and pH (using a calibrated, glass-probe pH meter) of thawed urine was subsequently measured for each rabbit at each level of cineole concentration. Animals were removed from the experiment if they lost >20 % of initial body weight. We removed 1 cottontail on day 10 (day 2 of

5 % cineole trial) and 1 pygmy rabbit on day 13 (day 2 of 7 % cineole trial).

Measuring Cineole in Food and Refusals The amount of cineole in the food offered and refused was quantified by grinding composite samples in liquid nitrogen and extracting 5 times with 2 ml pentane. We used capillary gas chromatography (GC, flame ionization detection, FID) to identify and quantify cineole with a Hewlett-Packard model 5890 Series II gas chromatograph (Palo Alto, CA, USA) using a 30-m× 0.25-mm id fused-silica capillary column with a 0.25-μm film of 5 % phenyl-95 % dimethylpolysiloxane (Zebron ZB-5, Phenomenex, Torrence, CA, USA). Cool on-column injection was used with oven programming from 40 °C to 200 °C at 10 °C/min, 200 °C to 300 °C at 40 °C/min. Helium was used as carrier gas and the FID was kept at 350 °C. We used a Hewlett-Packard 6890 Series GC-Mass Spectrometer (GC-MS, similar separation conditions, spectra collected at 70 eV and analyzed using Hewlett-Packard Chemstation software) for product identification by comparing retention times and mass spectra to authentic 1,8-cineole and for further quantitation by using standard curves of the authentic standard.

Measuring Cineole and Cineole Metabolites in Urine and Feces We determined the total amount of free and conjugated cineole metabolites in the urine of pygmy rabbits and cottontails consuming 0, 1, 3, 5, and 7 % cineole diets by using a 2-step procedure following that of Boyle et al. (2000). Samples were run in triplicate. Each sample was enzymatically hydrolyzed, acidified, and extracted twice with 1.5 ml ethyl acetate, with terpinen-4-ol added as an internal standard. Following reduction of the organic phases, methyl ester- and trimethylsilyl derivitization was performed using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and chlorotrimethyl silane (TMCS) as the catalyst according to manufacturer's instructions (Alltech Associates, Deerfield, IL, USA). Cineole metabolites were analysed with GC and GC-MS using authentic standards of 2-, 3-, 9-, and 7-hydroxycineole, 7- and 9-cineolic acid, and 7-hydroxy, 9-cineolic acid that were similarly treated (Table 1, Fig. 2). Tentative identification of cineole metabolites for which no standards were available was based on polarity (retention times) and mass spectra (parent ions and characteristic mass peaks).

To determine the relative proportions of free and conjugated metabolites in both urine and feces, we conducted a two-step procedure using urine (0.5 ml) and feces (0.5 g suspended in 2.5 ml acetate buffer, pH 7) from 4 rabbits of each species that consumed 5 % cineole. Free metabolites were removed by ethyl acetate extraction whereas the remaining conjugated metabolites (in the aqueous extract) were released by enzymatic hydrolyzation; both samples were reduced and derivatized, as described in the previous paragraph, before analysis by GC and GC-MS.

Measuring Unmetabolized Cineole in Feces To measure the amount of unmetabolized cineole in the rabbits' feces, we used headspace analysis on feces from both cineole-free and 5 % cineole diets, because it offered a more sensitive approach for detecting low concentrations of a highly volatile compound. Wet feces were ground, and 100 mg were placed into a 20-ml glass headspace autosampler vial and sealed. A cineole standard was prepared by adding 5 μl pure cineole to 100 mg (wet weight) of control feces obtained from rabbits on control diets and sealing the vial. Samples were incubated at 100 °C for 30 min in an Agilent 7694 headspace sampler (Agilent Technology, Santa Clara, CA). One ml of headspace gas was injected into a J&W DB-5 capillary column (30 m×250 μm×0.25 μm) installed in an Agilent 6890N GC. Splitless injection mode was used and injector temperature was 250 °C. The flow rate of helium as carrier gas was 1 ml/min. The flame ionization detector was maintained at 300 °C. Chromatographic separation was achieved with a temperature program initially held at 40 °C for 2 min and increased to 150 °C at 15 °C/min and held for 10 min.

Glucuronic Acid and Energy Content of Urine We measured the production of glucuronic acid (μmol/ml) in the urine of 4 pygmy rabbits and 4 cottontails on cineole-free diets and diets containing 5 % cineole by using the methods of Blumenkranz and Asboe-Hansen (1973). We measured the gross energy content (j/g) of 1–3 ml urine soaked in cotton from the cineole-free diets and diets containing 5 % cineole by using a bomb-calorimeter (IKA C-5000, IKA Works, Inc., Wilmington, NC, USA).

Statistical Analyses All data were reported as mean ± SE unless otherwise indicated. We examined the effects of cineole concentration and rabbit species on dry matter ($g/d/kg^{0.75}$) and cineole ($mmol/d/kg^{0.75}$) intake, production, and composition of urinary metabolites (μmol/g DM intake/day, μmol/μmol cineole ingested/day), and urine pH using a two-way repeated measures mixed linear model with interactions (PROC MIXED, SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). For significant effects of cineole concentration and interactions, we compared least squares means by using *post hoc* pairwise analyses with $\alpha=0.05$. We compared the proportion of free metabolites (%) in the urine among metabolite types and rabbit species using a two-way analysis of variance with interactions (PROC GLM). We compared the proportion of fecal metabolites (% of total) within pygmy rabbits using a one-way ANOVA, and composition of cineole metabolites between urine and feces in pygmy rabbits using a paired *t*-test, after checking for equal variances (PROC TTEST). We compared the increase in glucuronic acid (μmol/μmol cineole/d) and gross energy (j/μmol cineole/d) in the urine between the cineole-free and 5 % cineole diets between the rabbit species by using a two-sample *t*-test.

Table 1 Retention time on capillary gas chromatography (GC) and GC-mass spectrophotometer (GC-MS) and percent detected (mean ± SE) of the internal standard, cineole and cineole metabolites recovered in urine and feces in pygmy rabbits (PR, *Brachylagus idahoensis*) and cottontail rabbits (CT, *Sylvilagus nuttalli*)

Group	Metabolite	Retention time		Standard available	Percent in urine		Percent in feces	
		(GC-MS)	(capillary GC)		PR	CT	PR	CT
Internal standard	Terpinen-4-ol	9.51	8.71	Yes	–	–	–	–
Cineole $C_{10}H_{18}O$	1,8-cineole	6.81	6.23	Yes	0	0	0	0
Hydroxycineoles $C_{10}H_{18}O_2$	3-hydroxycineole	10.28	9.96	Yes	3.0±1.3	0.7±0.2	21.8±3.8	0
	2-hydroxycineole	10.91	10.37	No	20.5±15.3	9.1±1.8	24.3±0.5	0
	9-hydroxycineole	11.30	11.00	Yes	39.2±8.4	60.6±1.7	32.5±2.8	0
	7-hydroxycineole	11.54	11.38	Yes	5.8±1.4	7.1±0.4	5.6±0.5	0
Cineolic acids $C_{10}H_{16}O_3$	9-cineolic acid	12.12	12.03	Yes	9.1±3.3	10.4±1.0	3.5±0.1	0
	7-cineolic acid	12.68	12.73	Yes	6.1±2.0	1.5±0.4	2.9±0.6	0
Major dihydroxycineoles $C_{10}H_{18}O_3$	Unknown A	13.87	13.54	No	3.2±1.0	1.6±0.2	0	0
	Unknown B	14.26	13.82	No	4.8±0.9	3.8±2.4	3.8±0.3	0
	Unknown C	14.52	14.14	No	7.9±1.3	5.4±0.6	5.6±0.3	0
Hydroxycineolic acids $C_{10}H_{16}O_4$	7-hydroxy-9-cineolic acid	15.43	15.18	Yes	0	0	0	0

Finally, we examined the effect of cineole intake on urine pH between rabbit species by using analysis of covariance (PROC GLM).

Results

Intake of Cineole Dry matter intake (g/d/kg$^{0.75}$) of pellets differed by cineole concentration ($F_{4,30}$=35.44, P<0.001) and by rabbit species ($F_{1,8}$=60.95, P<0.001), and the cineole concentration × species interaction also was significant ($F_{4,30}$=16.97, P<0.001). Dry matter intake of pygmy rabbits remained constant until cineole concentration reached 7 %, whereas dry matter intake of cottontails declined at the lowest level of cineole offered (1 %), and continued to decline as cineole concentration increased (Fig. 3a). Pygmy rabbits consumed more pellets in relation to metabolic body mass at all cineole concentrations than did cottontails (Fig. 3a). Voluntary intake of cineole (mmol/d) through pellet consumption varied with cineole concentration ($F_{4,30}$=147.32, P<0.001) and rabbit species ($F_{1,8}$=55.84, P<0.001), and the cineole concentration × species interaction also was significant ($F_{4,30}$=40.37, P<0.001). Cineole intake increased with cineole concentration in the pellets for pygmy rabbits, peaking at 5 % cineole (Fig. 3b). Cineole intake of cottontails also increased with cineole concentration, but at a lower rate than pygmy rabbits (Fig. 3b). Pygmy rabbits voluntarily consumed 2–4 times more cineole in relation to metabolic body mass than cottontails at all concentrations of cineole (Fig. 3b). The average loss of cineole from pellets from the time fed to the time refusals were collected was 11.1 % (SD=1.9 %).

Cineole Metabolites in Urine and Feces No unmetabolized 1,8-cineole was detected in the urine or feces of either pygmy rabbits or cottontails when consuming diets of any cineole concentration. We classified cineole metabolites detected in the urine and feces according to the extent of oxidation they had undergone and the functional groups they acquired during oxidation (i.e., hydroxycineoles = 1 oxygen, cineolic acids = 2 oxygens, dihydroxycineoles = 2 oxygens, and hydroxycineolic acids = 3 oxygens, Table 1, Fig. 2). We confirmed the presence of 2-, 3-, 7-, and 9-hydroxy cineole, and 7- and 9-cineolic acid, which had been previously identified in brushtail possums consuming cineole (Boyle et al., 2000), in the urine from both species of rabbits and in the feces of pygmy rabbits (Table 1). However, we did not find 7- hydroxy-9 cineolic acid, or many other unidentified dihydroxycineoles and hydroxycineolic acids also found in possums (Boyle et al., 2000), in the urine or feces of either rabbit species. We also detected 3 major unidentified cineole metabolites (dihydroxycineoles A, B, and C) in the urine of both pygmy rabbits and cottontails, which were most likely dihydroxycineoles based on their mass spectra and parent ions (Boyle et al., 2000). However, only dihydroxycineoles B and C were detected in the feces of pygmy rabbits (Table 1). Using the GC-MS, we detected up to 5 additional minor peaks that may have been dihydroxycineole or hydroxycineolic acids, but quantitation and absolute identification were not possible

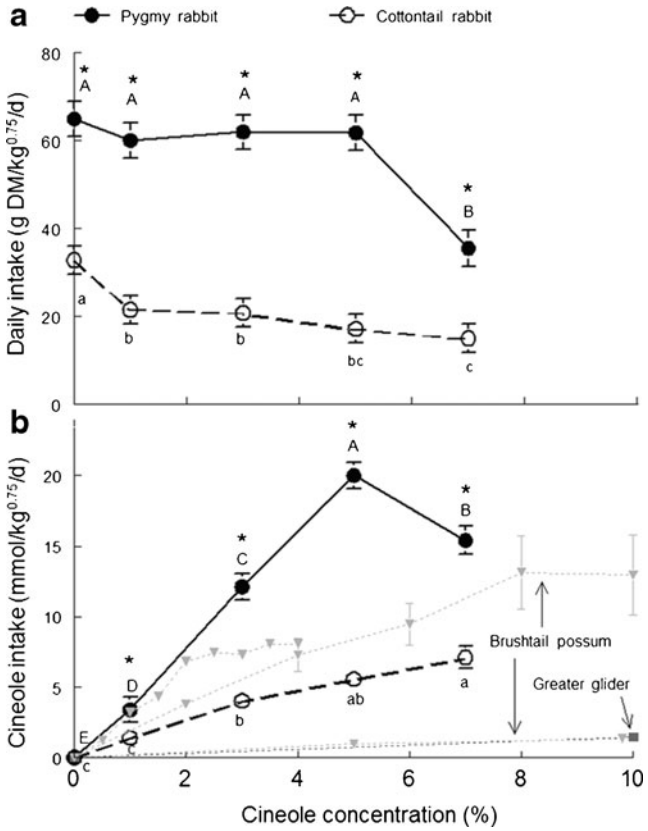

Fig. 3 Mean daily dry matter (DM) intake **a** and cineole intake **b** in relation to metabolic body mass in pygmy rabbits (*Brachylagus idahoensis*, *solid circles and line*, *capital letters*) and cottontails (*Sylvilagus nuttalli*, *open circles* and *dashed line*, *lower case letters*) as a response to cineole concentration of a pelleted diet. Intake in relation to metabolic body mass for brushtail possums (*Trichosurus vulpecula*, *gray diamonds*, Lawler et al., 1999; Boyle and McLean, 2004; DeGabriel et al., 2009) and greater gliders (*dark gray square*, *Petauroides volans*, Lawler et al., 1999) are provided for comparison. Each *line* represents a distinct study. *Different letters* denote significant differences among cineole concentrations within rabbit species and *asterisks* denote a significant difference between pygmy rabbits and cottontails for each cineole concentration (α=0.05)

because of low quantities. An average of 28.1±4.3 % of ingested cineole was recovered as cineole metabolites in the urine and 3.8±0.3 % as metabolites in feces of pygmy rabbits, whereas only 15.7±4.2 % was recovered in the urine, and no metabolites were recovered in the feces of cottontail rabbits. Therefore, we recovered twice as much of the ingested cineole in pygmy rabbits than in cottontails (t=2.79, df=6, P=0.02).

The total amount (μmol) of cineole metabolites produced in the urine in relation to food consumed increased with increasing cineole in the diet for both rabbit species ($F_{3,17}$=10.05, P<0.001, Fig. 4a). Each individual cineole metabolite (P<0.05), and the amount of all hydroxycineoles ($F_{3,17}$=10.24, P=0.02), cineolic acids ($F_{3,17}$=56.45, P<0.001), and dihydroxycineoles ($F_{3,17}$=28.16, P<0.001), increased in the urine as the cineole concentration in the diet increased (Fig. 4a). However, the total amount of cineole metabolites, groups of metabolites, and individual cineole metabolites produced per μmol of cineole consumed remained constant with increasing concentration of cineole in the diet (P>0.05). Although pygmy rabbits tended to produce more total metabolites ($F_{1,6}$=4.24, P=0.08) and hydroxycineoles ($F_{1,6}$=4.66, P=0.07) in relation to ingested cineole than did cottontails (Fig. 4b), differences were not statistically significant. However, pygmy rabbits produced 4–5 times more cineolic acids ($F_{1,6}$=5.75, P=0.05), 3-hydroxy- ($F_{1,6}$=11.02, P=0.02), 2-hydroxycineole ($F_{1,6}$=15.54, P=0.008), and 7-cineolic acid ($F_{1,6}$=27.99, P=0.002) per μmol cineole consumed than did cottontails, and there was a cineole concentration×species interaction for dihydroxycineole A ($F_{3,17}$=8.02, P=0.002) and C ($F_{3,17}$=3.73, P=0.03).

Extent of Oxidation of Cineole Metabolites The proportion of each individual metabolite, and groups of metabolites, in the urine remained constant across all cineole concentrations (all P>0.05, Fig. 4b), and there were no species×cineole concentration interactions (all P>0.05). In their urine, pygmy rabbits ($\overline{X} = 69.8 \pm 3.1\%$) and cottontails ($\overline{X} = 77.3 \pm 0.7\%$) produced the highest proportion of hydroxycineoles, the least oxidized cineole metabolites with one added oxygen, followed by cineolic acids (pygmy rabbits: $\overline{X} = 14.9 \pm 2.6\%$: cottontails: $\overline{X} = 12.0 \pm 0.7\%$), and dihydroxycineoles, both with 2 added oxygens (pygmy rabbits: $\overline{X} = 15.4 \pm 0.6\%$, cottontails: $\overline{X} = 10.7 \pm 1.0\%$, Table 1, Fig. 5). As in their urine, pygmy rabbits produced the greatest proportion of their fecal metabolites as hydroxycineoles ($\overline{X} = 84.3 \pm 0.4\%$, ($F_{1,7}$=52.57, P<0.001), with much lower proportions of cineolic acids ($\overline{X} = 6.4 \pm 0.6\%$) and dihydroxycineoles ($\overline{X} = 9.3 \pm 0.5\%$, Table 1, Fig. 5). Fecal metabolites comprised 19.8±3.8 % of the total metabolites, 23.6±5.4 % of the total hydroxycineoles, 8.9±1.4 % of the total cineolic acids, and 12.9±2.8 % of the total dihydroxycineoles produced by pygmy rabbits in the urine and feces combined (Table 1, Fig. 6).

Pygmy rabbits produced a 30 % greater proportion (30±3.1 %) of more highly-oxidized cineole metabolites (cineolic acids and dihydroxycineoles) relative to less-oxidized metabolites (hydroxycineoles) in their urine than did cottontails (22.7±0.7 %, t=2.43, df=6, P=0.04). Specifically, 3-hydroxycineole (t=3.50, df=6. P=0.04), 2-hydroxycineole (t=7.13, df=6, P<0.001), and 7-cineolic acid (t=4.57, df=6, P=0.02) composed a greater proportion of total metabolites in pygmy rabbits than in cottontails, whereas 9-hydroxycineole (t=5.07, df=6, P=0.01), and dihydroxycineole C (t=4.20, df=6, P=0.006) composed a greater proportion of total

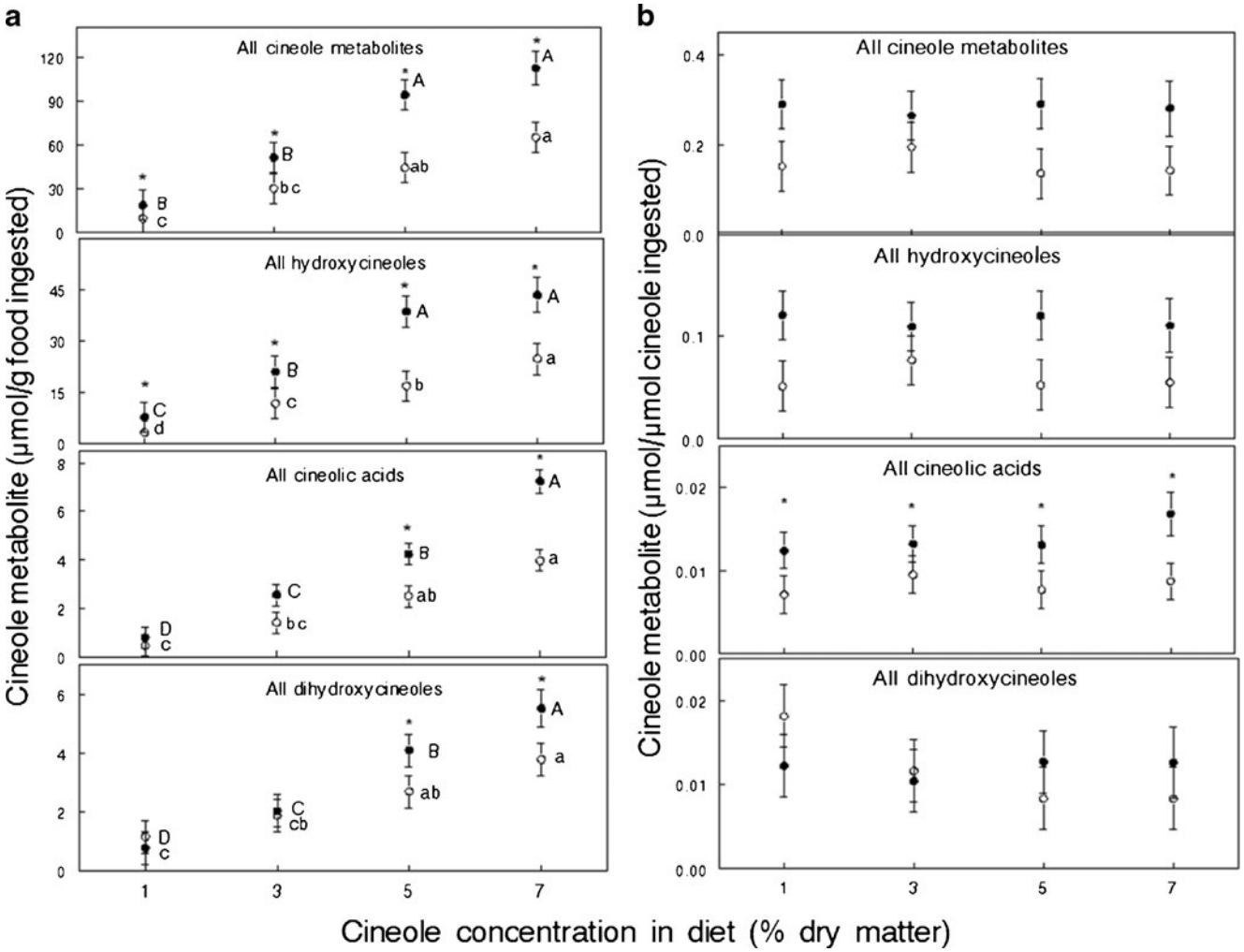

Fig. 4 Mean amount of cineole metabolites in urine produced per day **a** relative to food pellet intake, and **b** relative to cineole ingested by pygmy rabbits (*Brachylagus idahoensis, solid circles*, *capital letters*) and cottontails (*Sylvilagus nutalli, open circles*, *lower case letters*) as the concentration of cineole increased in their pelleted diet. *Different letters* denote significant differences among cineole concentration within rabbit species, and *asterisks* denote significant differences between rabbit species (α=0.05)

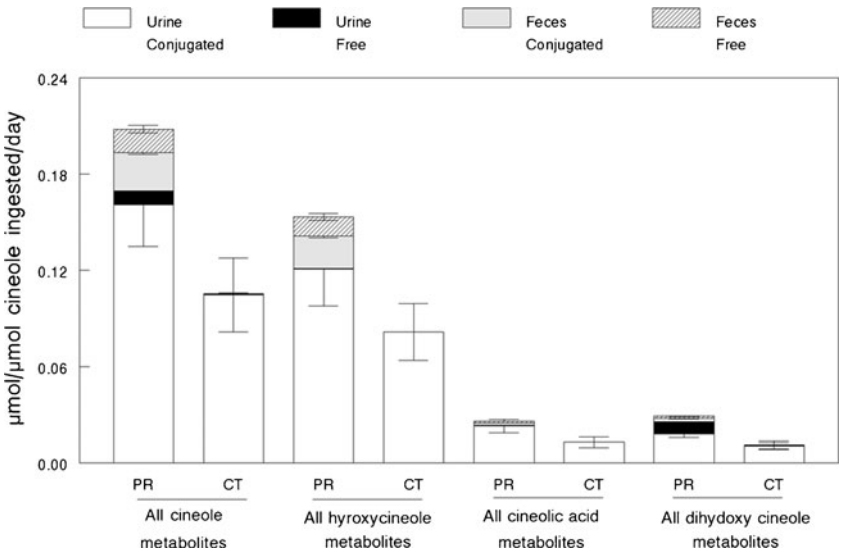

Fig. 5 The amount of conjugated and free cineole metabolites produced in the urine and feces of pygmy rabbits (PR, *Brachylagus idahoensis)* and cottontails (CT, *Sylvilagus nuttalli)* per day when rabbits were consuming pellets containing 5 % cineole

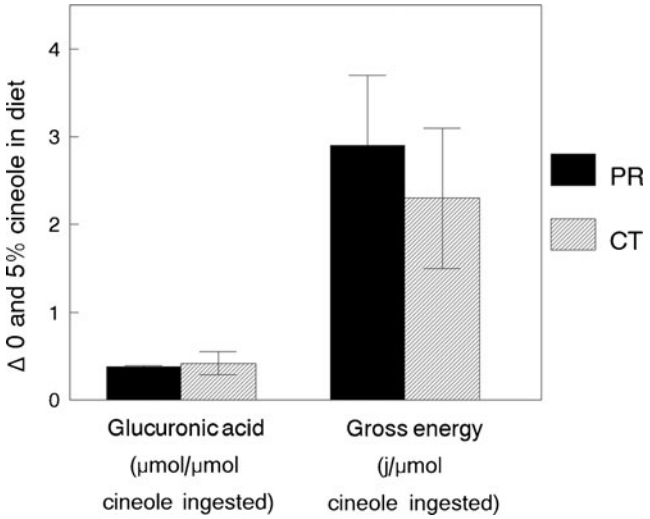

Fig. 6 Change in glucuronic acid and gross energy in the urine of pygmy rabbits (PR, *Brachylagus idahoensis*, *solid bars*) and cottontail rabbits (CT, *Sylvilagus nuttalli*, *hatched bars)* between diets of cineole-free (0 %) food pellets and pellets with 5 % cineole concentration in relation to cineole ingested. Glucuronic acid and gross energy in urine did not differ between rabbit species (α=0.05)

metabolites in cottontails than in pygmy rabbits. Fecal metabolites of pygmy rabbits were less oxidized than urinary metabolites, with a significantly lower proportion of cineolic acids and dihydroxycineoles ($\overline{X} = 15.6 \pm 0.3\%$) than in the urine ($\overline{X} = 22.5 \pm 0.6\%$, t=4.85, df=6, P=0.003).

Extent of Conjugation of Cineole Metabolites The proportion of cineole metabolites in the urine that were free (unconjugated) rather than conjugated varied with specific metabolite ($F_{2,\ 24}$=242.74, P<0.001) and rabbit species ($F_{1,\ 24}$=156.52, P<0.001), and the species × metabolite interaction was significant ($F_{1,\ 24}$=120.76, P<0001). A greater proportion of dihydroxycineoles in the urine was free (pygmy rabbits: $\overline{X} = 30.0 \pm 0.7\%$; cottontails: $\overline{X} = 5.5 \pm 1.6\%$) than of cineolic acids (pygmy rabbits: $\overline{X} = 1.5 \pm 0.1\%$; cottontails: $\overline{X} = 1.5 \pm 0.1\%$) and hydroxycineoles (pygmy rabbits: $\overline{X} = 2.0 \pm 0.1\%$; cottontails: $\overline{X} = 0 \pm 0\%$) in both pygmy rabbits ($F_{2,\ 11}$=375.4, P<0.001) and cottontails ($F_{2,\ 11}$=10.19, P=0.005, Fig. 5). Pygmy rabbits produced an average of 5.2±0.3 % free metabolites in urine overall, a proportion 6 times greater than that of cottontails, which averaged only 0.7±0.1 % free metabolites (t=14.58, df=6, P<0.001). More specifically, pygmy rabbits produced a greater proportion of free hydroxycineoles ($F_{1,\ 7}$=42.42, P<0.001) and free dihydroxycineoles ($F_{1,7}$=193.1, P< 0.001) in their urine than did cottontails (Fig. 5). The proportion of cineole metabolites that were free in the feces of pygmy rabbits averaged 37.3±3.6 %; but did not differ among metabolite types ($F_{2,\ 6}$=2.73, P=0.14, Fig. 5). However, a greater proportion of fecal metabolites vs. urinary metabolites was free (t=10.65, df=5, P<0.001, Fig. 5).

Glucuronic Acid, Gross Energy, and Urine pH Pygmy rabbits and cottontails had a similar increase in the amount of glucuronic acid (μmol/μmol cineole/ d, t=0.28, df=6, P= 0.80) and gross energy (j/μmol cineole/d, t=0.55, df=5, P= 0.61) in the urine when consuming 5 % cineole diet compared to the cineole-free diet (Fig. 6). Likewise, urine pH differed by cineole concentration ($F_{4,\ 27}$=14.10, P<0.001), but not by rabbit species ($F_{1,8}$=0.40, P=0.51) nor was there a cineole × species interaction ($F_{4,27}$=0.14, P=0.97). pH remained constant as cineole increased from 0 % to 3 %, but was significantly lower for both species at cineole concentration of 5 and 7 % than at other concentrations (Fig. 7a). Similarly, pH declined with increasing cineole intake (mmol/d), ($F_{1,44}$=12.87, P<0.001) but this relationship did not differ between rabbit species ($F_{1,44}$=0.69, P=0.41), nor was there a cineole × species interaction ($F_{1,44}$=1.47, P=0.23, Fig. 7b).

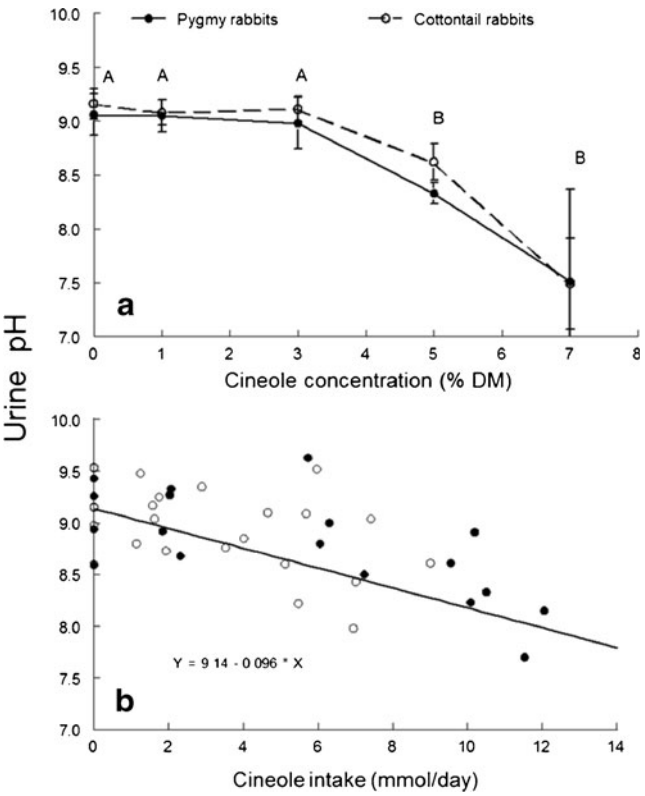

Fig. 7 The relationship between urine pH and **a** cineole concentration of the pelleted diet, and **b** amount of cineole ingested by pygmy rabbits (*Brachylagus idahoensis*, *closed circles*) and cottontail rabbits (*Sylvilagus nuttalli*, *open circles*). *Different letters* denote significant differences in pH among cineole concentrations. pH did not differ between rabbit species (α=0.05)

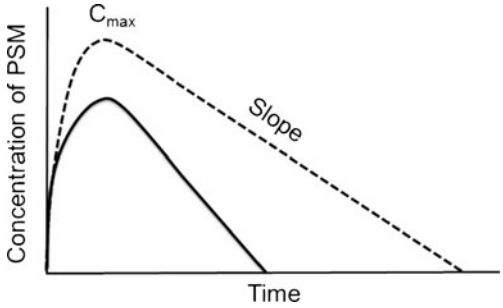

Fig. 8 A theoretical schematic of the systemic concentration-time course of cineole for pygmy rabbits (*Brachylagus idahoensis*, *solid line*) and cottontails (*Sylvilagus nuttalli*, *dashed line*) following ingestion of the same dose of cineole. The proposed lower absorption of ingested cineole in the intestine by pygmy rabbits would result in a lower maximum concentration (C_{max}). The proposed faster or more complete excretion of cineole metabolites by pygmy rabbits would increase the elimination rate (slope) of cineole. Both of these proposed mechanisms would lower the overall systemic exposure (area under the concentration-time curve) to cineole for pygmy rabbits compared to cottontails

Discussion

Compared to generalist cottontails, pygmy rabbits, which specialize on diets of sagebrush that contain high levels of monoterpenes, were able to consume and detoxify high levels of cineole, by using many, but not all, of the hypothesized strategies (Fig. 1). Pygmy rabbits were able to maintain their intake of pellets until cineole content exceeded 5 %, nearly twice the total monoterpene content of sagebrush (White et al., 1982; Shipley et al., 2006), whereas cottontails immediately reduced intake at only 1 % cineole (Fig. 1). As a result, pygmy rabbits consumed a maximum of about 20 mmol of cineole/d/$kg^{0.75}$ for 3 days. In marsupial folivores, both a generalist (brushtail possum, Lawler et al., 1999; Boyle and McLean, 2004; DeGabriel et al., 2009) and a specialist (greater glider, *Petauroides volans*, Lawler et al., 1999) of *Eucalyptus*, which also contains high levels of cineole, began reducing their intake when fed diets of 2–3 % cineole (Fig. 3a).

Pygmy rabbits, like cottontails, brushtail possums, and koalas (specialists on *Eucalyptus*, Boyle et al., 2001), did not avoid absorption and detoxification of cineole by excreting it unchanged in their feces as we had hypothesized (Fig. 1). In contrast, Stephens woodrats (*Neotoma stephensi*) that specialize on one-seeded juniper (*Juniperus monosperma*) had a concentration of the monoterpene α-pinene 5 times lower in their bloodstream (Sorensen and Dearing, 2003) and excreted 3 times more α-pinene in their feces when consuming juniper and α-pinene than did the generalist woodrat (*N. albigula*, Sorensen et al., 2004). However, pygmy rabbits did have an unexpectedly high concentration of metabolized cineole in their feces, whereas none was detected in the feces of our cottontails or in koalas (Boyle et al., 2001). Although cineole metabolites also have been found in brushtail possum feces, they constituted only 6.5 % of all cineole metabolites (Boyle et al., 2000) compared to nearly 20 % in pygmy rabbits (Fig. 5). Fecal metabolites represented a major avenue of elimination of cineole in pygmy rabbits, which has been a hypothesized (Sorensen and Dearing, 2006, Fig. 1), but previously undocumented, difference between specialist and generalist herbivores.

Cineole metabolites in the feces of pygmy rabbits may have originated from microbial, intestinal, or hepatic detoxification (Fig. 1). For example, monoterpenes are readily metabolized by fiber-adapted microflora in the rumens of Mediterranean dairy goats (Malecky and Broudiscou, 2009). Microbes also inhabit the cecum and the mucosal lining of the intestine before the cecum in many animals (Yamamoto et al., 2009; Angert et al., 2010) and, therefore, may contribute to detoxification of PSMs before absorption in hindgut fermenting-herbivores like pygmy rabbits. The location and function of intestinal microbes deserves the recent attention by ecologists as a mechanism of tolerance to PSMs in herbivores (Kohl et al., 2011).

Alternatively, metabolites of cineole in the feces of pygmy rabbits may be formed by detoxification enzymes in the liver and excreted back into the intestine via the bile or formed by detoxification enzymes in the enterocytes of the gut. Several PSMs are excreted primarily from the liver into the bile and excreted in the feces (Grigoleit and Grigoleit, 2005; Barnes et al., 2011). In addition, relatively high activity of the enzyme Cytochrome P450 3A (CYP3A), which metabolizes monoterpenes like 1,8-cineole (Miyazawa et al., 2001), has been found in the duodenal tissue of brushtail possums (Sorensen et al., 2007). The role of these detoxification enzymes in the intestine in facilitating tolerance to PSMs deserves further attention.

Despite a relatively large amount of cineole metabolites in the feces, the majority of cineole ingested by pygmy rabbits was presumably detoxified in the liver and excreted in the urine. Although only 3- and 2-hydroxycineoles have been identified previously in domestic rabbit urine (Miyazawa et al., 1989), pygmy rabbits and cottontails produced all of the hydroxycineoles and cineolic acids identified in brushtail possum urine, but few of the dihydroxycineoles and no hydroxycineolic acids. We did not detect an induction of detoxification pathways in the rabbits during our trial, because excretion rate relative to cineole ingested and composition of metabolites remained constant over 15 days and 4 levels of cineole in the diet (Fig. 4b).

To a limited degree, pygmy rabbits excreted cineole metabolites that were more oxidized and less conjugated than did cottontails, and thus they used less energetically expensive methods to detoxify cineole, as we had hypothesized (Fig. 1). Both rabbit species produced mono- and

dioxygenated metabolites, but pygmy rabbits excreted over 30 % more metabolites as dioxygenated compounds than did cottontails. However, both rabbit species excreted much less of the highly-oxidized cineole metabolites in their urine than did the generalist brushtail possum and the specialist koala (Boyle et al., 2000, 2001). Brushtail possums and koalas excreted between 60 % and 85 % of the trioxygenated cineole metabolites (i.e., hydroxycineolic acids), and 9 % of the koala's urinary metabolites were tetraoxygenated (i.e., dicineolic acids), neither of which were detected in the urine or feces of either rabbit species. Also, brushtail possums excreted less of the least oxidized metabolites (hydroxycineoles) in the feces than urine, whereas pygmy rabbits excreted more of the least oxidized metabolites in the feces than urine.

Similarly, pygmy rabbits excreted a greater proportion (5 %) of their urinary metabolites in the unconjugated, free form, than did cottontails (0.7 %), and over a third of their fecal metabolites were free. In comparison, brushtail possums have been shown to excrete over 3/4 of their urinary metabolites, and all of their fecal metabolites, in the free form (Boyle et al., 2000). Thus, both rabbit species used the relatively more energetically expensive methods of detoxification (conjugation vs. oxidation) compared to the *Eucalyptus*-eating marsupials. Although conjugation is thought to be more energetically expensive than oxidation (Boyle et al., 1999, 2000), it generally proceeds faster and results in metabolites that are more readily excreted than oxidation reactions (Casarett et al., 2008). Moreover, conjugation may not pose a large energetic cost if substrates for these pathways are readily available in the diet (Dash, 1988). Because they were able to consume and metabolize greater quantities of 1,8-cineole than cottontails, and even marsupial folivores, yet produced similar amounts of urinary energy and glucuronic acid relative to cineole consumption, pygmy rabbits seem to rely on capacity rather than efficiency for coping with monoterpenes in their diet.

We recognize that we measured the fate of only one of the many PSMs in sagebrush. Cineole is not necessarily the most toxic PSMs in sagebrush nor the most difficult to eliminate. Other monoterpenes such as artemiseole and methacrolein recently have been identified as influencing diet selection by pygmy rabbits more than cineole (Ulappa, 2011). Identification of the detoxification pathways of other PSMs in sagebrush deserves additional attention.

Our relatively low (~46 %) recovery of cineole in urine and feces suggests that we may have missed some important fates of ingested cineole. First, we may have overestimated intake of cineole in our rabbits. Approximately 11 % of the cineole added to the food pellets evaporated over the 8-h periods between refreshing the pellets offered, and this loss could account for a small portion of the cineole not recovered. If present, a small amount of unmetabolized cineole also may have volatilized from fecal pellets during the day and further lowered recovery. In addition, some of the cineole likely volatilized during chewing and was absorbed and released in the breath of rabbits. White et al. (1982) found that more than 90 % of the expected cineole in sagebrush consumed by wild pygmy rabbits was lost, presumably from the breath, by the time the ingesta reached the stomach. Similar losses of volatile monoterpenes from the contents of the upper intestine compared to the diet have been observed in sage grouse (Welch et al., 1989) and mule deer (Cluff et al., 1982). However, these studies did not quantify the amount of monoterpenes consumed, nor did they account for possible absorption or detoxification of monoterpenes before the small intestine.

An additional explanation for our low recovery of cineole may be caused by recycling of metabolized cineole through enterohepatic recirculation (Fig. 1, Grigoleit and Grigoleit, 2005) or ingestion of cecal droppings. Both of these processes could slow the elimination of ingested cineole and reduce its recovery. Enterohepatic recirculation may explain why concentrations of metabolites of cineole consumed by brushtail possums were highest in the feces two days after feeding of cineole stopped (Boyle et al., 2000). Unlike brushtail possums, rabbits are cecatrophic, and this behavior could further lengthen the time for completely eliminating cineole. Future studies should measure urinary and fecal excretion of cineole until metabolites are no longer detected, and under conditions where cecal droppings can be investigated.

Finally, we may not have detected some cineole metabolites because they may have been conjugated to another polar molecule. Although glucuronidation is the major pathway of detoxification in mammals (Casarett et al., 2008), it may receive biased attention from ecologists because glucuronidated metabolites are relatively easy to measure (Blumenkranz and Asboe-Hansen, 1973). Many substrates that undergo glucuronidation also undergo sulfonation, and several monoterpenes can be sulfonated (Welniak, 2003). Monoterpenes also may be conjugated to amino acids such as glycine, glutamine, taurine, serine, and proline and the tripepetide glutathione (Casarett et al., 2008). Several of the enzymes responsible for these alternative conjugation pathways can be induced in herbivores following ingestion of PSMs and can differ among dietary specialists and generalists (Haley et al., 2007; Skopec et al., 2007). Results from these *in vitro* assays indicate a need to use advanced bioanalytical techniques (e.g., Liquid Chromatography and Mass Spectrometry) to investigate a wider range of conjugation metabolites excreted by herbivores consuming PSMs.

In summary, this study suggests that pygmy rabbits have a greater capacity to minimize systemic exposure to cineole than cottontails by minimizing absorption and maximizing detoxification of ingested cineole. Although cineole was not excreted unchanged in the feces, excretion of its metabolites

in the feces formed by either microbial or rabbit enzymes in the gastrointestinal tract represents lower absorption, and thus lower systemic exposure to cineole (per dose consumed, Fig. 8). Second, greater excretion of cineole metabolites in urine relative to cineole dose by pygmy rabbits compared to cottontails would further lower systemic concentration of unchanged cineole and its metabolites. However, mechanisms that lower systemic exposure to cineole may come with a higher cost in pygmy rabbits than in cottontails in the form of excretion of glucuronic acid and energy. We propose that the benefits associated with energetically expensive mechanisms that minimize absorption and maximize detoxification and excretion allow pygmy rabbits to specialize on chemically defended sagebrush.

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Possible Ecological Role of Pseudopterosins G and P-U and SECO-Pseudopterosins J and K from the Gorgonian *Pseudopterogorgia elisabethae* from Providencia Island (SW Caribbean) in Regulating Microbial Surface Communities

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Abstract The gorgonian *Pseudopterogorgia elisabethae* collected at Providencia Island (Colombia) has an unfouled surface, free of obvious algal and invertebrate growth. This gorgonian produces significant amounts of the glycosilated diterpenes pseudopterosins and *seco*-pseudopterosins (Ps and *seco*-Ps). Our previous experiments have shown activity of these compounds against eukaryotic (human cancer cell lines and *Candida albicans*) and prokaryotic cells (*Staphylococcus aureus* and *Enterococcus faecalis*). However, the potential role of pseudopterosins on the regulation of the fouling process is still under study. We evaluated the activity of these compounds against bacteria isolated from heavily fouled marine surfaces as an indicator of antifouling activity. Additionally, we assessed their activity against bacteria isolated from *P. elisabethae* to determine whether potentially they play a role in preventing surface bacterial colonization, thus impairing presumptively the establishment of further successional stages of fouling communities. Results showed that Ps and *seco*-Ps seem to modulate bacterial growth (controlling Gram-positive bacterial growth and inducing Gram-negative bacterial associations). We thus hypothesized that Ps and *seco*-Ps may play a role in controlling microbial fouling communities on the surface of this gorgonian. By using bTEFAP and FISH we showed that the most abundant bacteria present in the microbial communities associated with *P. elisabethae* are Gram-negative bacteria, with Proteobacteria and Gammaproteobacteria the most representative. To evaluate whether Ps and *seco*-Ps have a direct effect on the structure of the bacterial community associated with *P. elisabethae*, we tested these compounds against culturable bacteria associated with the surface of *P. elisabethae*, finding remarkable selectivity against Gram-positive bacteria. The evidence presented here suggests that Ps and *seco*-Ps might have a role in the selection of organisms associated with the gorgonian surface and in the regulation of the associated bacterial community composition.

Keywords *Pseudopterogorgia elisabethae* · Pseudopterosins and *seco*-pseudopterosins · Ecological role · Bacterial community · Biofilm inhibition · Antibacterial

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Introduction

Gorgonian octocorals are marine organisms that produce a broad array of novel natural products with promising anti-inflammatory, anticancer, antituberculosis, antimicrobial, and antifouling activities (Blunt et al. 2011). Among those organisms, *Pseudopterogorgia elisabethae*, which can be abundant at certain Caribbean locations, has been the source of several compounds with diverse biological applications, particularly the diterpene glycosides called pseudopterosins

(Ps) and *seco*-pseudopterosins (*seco*-Ps) (Heckrodt and Mulzer 2005; Marrero et al. 2010). Recently, we isolated diterpene glycosides from specimens of *P. elisabethae* collected at Providencia Island, Colombia (SW Caribbean Sea), including the previously unknown types PsP-V and *seco*-PsK and 5 known compounds PsG, PsK, *seco*-PsJ, and amphilectosins A and B (Duque et al. 2004, 2006; Puyana et al. 2004). Rodriguez et al. (2004) reported the same chemical compositions for specimens collected at the same location but with 5 additional new compounds: PsW, 2′-*O*-acetyl-PsQ, 3′-*O*-acetyl-PsQ, 2′-*O*-acetyl-PsU, and 3′-*O*-acetyl-PsU. Some of these compounds exhibited pharmacological properties including antibacterial, anti-inflammatory, anticancer, antiviral, anti-malarial, and anti-tuberculosis activities (Rodriguez et al. 2004; Correa et al. 2009, 2011).

Our previous reports on the chemical composition of the Colombian *P. elisabethae* collected at Providencia Island (Duque et al. 2004, 2006) demonstrated that the structures of Ps and *seco*-Ps are quite different from those isolated from specimens collected at other Caribbean locations (different sugar moieties, glycosylation position, and aglycone stereochemistry). Not only is the chemical composition different among various populations of *P. elisabethae*, but pseudopterosin concentration also is highly variable. For example, colonies from Providencia Island have pseudopterosin concentrations ranging between 11 % and 16 % dry mass compared to a 4 % of total pseudopterosin content in specimens from the Bahamas (Look et al. 1986a, b; Puyana et al. 2004). Despite their importance, little is known about the ecological role of pseudopterosins and related compounds. O'Neal and Pawlik (2002) evaluated the feeding deterrent properties of crude organic extracts from 32 species of Caribbean gorgonians, where *P. elisabethae* was invariably deterrent. Although no chemical characterization of the extracts was done, it is likely that the extracts evaluated were rich in pseudopterosins since the chemical composition of Bahamian specimens is quite consistent (Look et al. 1986a). Thornton and Kerr (2002) reported an increase in the biosynthesis of PsC in response to high levels of predation by the gastropod *Cyphoma gibbosum* and in response to decreased levels of UV/visible radiation.

Our field observations (Paz 2008) have shown that *P. elisabethae* is invariably free of obvious algal and invertebrate fouling. Ps and *seco*-Ps from specimens from Providencia Island also showed selective activity against pathogenic Gram-positive bacteria (Correa et al. 2011). These observations lead us to the question whether there is a relationship between the selective activity of Ps and *seco*-Ps and the surface microbial composition of *P. elisabethae*. We thus hypothesized that Ps and *seco*-Ps may play a role in controlling microbial fouling communities on the surface of this gorgonian. Thus, the *P. elisabethae* holobiont (the macroorganism and their associated microorganisms such as endosymbiotic algae, protists, fungi, bacteria, Archaea, and viruses, as well as the symbioses among them) may use Ps and *seco*-Ps to control the attachment of bacteria and other microorganisms to its surface.

In order to evaluate whether Ps and *seco*-Ps isolated from *P. elisabethae* may influence the selection, settlement, and colonization of microorganisms, we determined the antifouling activity of Ps and *seco*-Ps against bacteria isolated from heavily fouled marine surfaces. We also evaluated the capacity of these compounds to interfere with bacterial biofilm formation on polystyrene surfaces. The challenge of bacteria to colonize a surface is based on the ability of those bacteria to establish themselves on a submerged substrate and form a biofilm. Some bacteria colonize mature biofilms but others have mechanisms to establish biofilms from the beginning (Harder et al. 2012). It is well-known that biofilm formation is the first step in the establishment of fouling communities. If chemical compounds inhibit bacteria that form biofilms, then biofilm development and the establishment of a subsequent fouling community could be prevented. Additionally, we characterized the bacterial community associated with the surface of *P. elisabethae* using FISH, and we compared these results with previous data obtained by pyrosequencing. Finally, we evaluated the antibacterial activity of Ps and *seco*-Ps against bacteria isolated from *P. elisabethae*'s own microbiota.

Methods and Materials

Octocoral Collection and Treatment Specimens of the octocoral *P. elisabethae* were collected by SCUBA (approximately 20–30 m depth) at the site El Planchón (13°25′0″N and 81°23′0″W″), Providencia Island, Colombia (SW Caribbean Sea) and identified by Prof. Monica Puyana by sclerites analysis following Bayer (1961). Voucher specimens coded as INV CNI 1612–1614 were deposited at the invertebrate collection of the Museo de Historia Natural Marina Colombiana (MHNMC) at the Instituto de Investigaciones Marinas de Punta Betín (INVEMAR), Santa Marta, Colombia. Three colonies were excised with sterilized scalpels and immediately placed in ziplock bags underwater. Samples were transported to the laboratory within 2 hr of collection and rinsed briefly with sterile artificial seawater (ASW).

Samples were divided into two halves. One part was used for FISH analysis and the other was dried for extraction and isolation of polar compounds. For FISH analysis, a small fragment (c. 2.0 cm per colony) was introduced into 15 ml falcon tubes with 5.0 ml of 20 % formaldehyde/50 % acetic acid.

Bacterial Strains and Growth Media Six marine bacterial strains, associated with heavily fouled surfaces from the

sponge *Aplysina lacunosa* and an empty shell of the bivalve *Donax* sp., previously isolated and identified by Mora-Cristancho et al. (2011), using 16S rDNA sequencing and taxonomic assignment by comparing "**p**" distances, were used for biofilm formation inhibition and disc-diffusion antibacterial assays. Species used in this work were *Ochrobactrum pseudogringnonense* strains 1 and 2, *Alteromonas macleodii* (Gram-negatives), *Kocuria* sp., *Bacillus* sp., and *Oceanobacillus iheyensis* (Gram-positives). Bacterial isolates were kept in slant cultures on Marine Medium Difco 2216 at 4 °C until use.

Pseudomonas putida IsoF, used as a model organism for biofilm formation, was kindly donated by Prof. Kathrin Riedel from the Department of Microbiology, Institute of Plant Biology, University of Zurich. The bacterium was kept in slant culture on Luria-Bertani (LB) medium, and it was used as a positive control in the biofilm formation assay.

Twenty-six culturable bacteria associated with the surface of *P. elisabethae*, previously isolated and identified by Correa et al. (unpublished), using 16S rDNA sequencing and taxonomic assignment by comparing "**p**" distances, were used for a disc-diffusion antibacterial assay. These bacteria belong to the phyla Actinobacteria (Gram-positive, 8 strains), Firmicutes (Gram-positive, 10 strains), and Proteobacteria (Gram-negative, 8 strains). The bacterial isolates were kept in slant cultures at 4 °C on Marine Medium Difco 2216 until use.

Isolation and Structure Elucidation of Polar Compounds from P. elisabethae Dried colony fragments (30 g) were extracted with a dichloromethane: methanol (1:1) mixture. The resulting extract was filtered and concentrated by rotary evaporation to obtain a dark-green oil (4.3 g). The isolation of each compound was conducted in accordance with our previously described procedures (Duque et al. 2004, 2006), with a few modifications. The crude extract (3.5 g) was subjected to flash C18 column chromatography and eluted with a sequence of solvents of decreasing polarity (500 ml each): methanol:water 1:9, fraction F1 (207.4 mg); methanol:water 1:1, F2 (247.8 mg); methanol:water 4:1, F3 (212.9 mg); methanol 100 %, F4 (1025.5 mg); ethanol 100 %, F5 (603.3 mg); acetone 100 %, F6 (485.5 mg); dichloromethane:methanol 1:1, F7 (433.7 mg); and dichloromethane 100 %, F8 (26.7 mg). Fractions F4 and F5 contained a clean mixture of Ps and *seco*-Ps as shown by LC-MS and TLC, thus they were mixed and renamed as FPST. Subsequently, part of FPST was separated by an automated flash chromatography system (Combiflash Rf, Teledyne Isco) using a diol column (150 g) with a continuous gradient mobile phase from hexane 100 % to hexane: ethanol (6:4) over 20 min with a 85.0 $ml\,min^{-1}$ flow rate. Ten fractions were collected (each ~250 ml) yielding PsG (19.1 mg), PsP (9.6 mg), PsQ (58.1 mg), PsS (2.3 mg), PsT (14.5 mg), PsU (46.6 mg), *seco*-PsJ (16.2 mg), and *seco*-PsK (1.2 mg). A final purification of all compounds was performed by HPLC using a Gemini C-18 column (5 μm, 10×250 mm) and methanol: water (9:1) as mobile phase with a 3.0 $ml\,min^{-1}$ flow rate. Isolated compounds were carefully identified by spectroscopic means (MS and ^{1}H and ^{13}C NMR, including DEPT), and the spectral data were compared with those obtained from our previous isolations of these compounds (Duque et al. 2004, 2006). Purity was confirmed by HPLC. Additionally, the relative natural content of Ps and *seco*-Ps in *P. elisabethae* was determined by LC-MS analysis of the crude extract using the total ion chromatogram trace (TIC). The quantification was performed using a calibration curve generated with pure PsA as a standard in various solutions of different concentrations ranging between 0.01 and 5.00 $mg\,ml^{-1}$. Quantities of individual Ps and *seco*-Ps were expressed relative to the dry mass of the animal prior to extraction (including gorgonian matrix and co-enenchyme).

Determination of Surface-Associated Bacteria by Fluorescence In Situ Hybridization (FISH) Fluorescence *in situ* hybridization (FISH) (Amann et al. 1995) was used to characterize the surface bacterial community associated with *P. elisabethae*. Correa et al. (unpublished) had previously used the same set of *P. elisabethae* samples to characterize the total bacterial community by using bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP). The bTEFAP data were used in this work in order to compare bacterial community characterizations by using two different approaches. For FISH analysis, the octocoral fragments were incubated in the fixing solution (formaldehyde 20 %/acetic acid 50 %) for 12 h. Fixed samples were scraped to obtain only surface-associated bacteria, and those were re-suspended in a phosphate buffered saline solution (PBS). Eight microliters from the fixed sample were dropped on a glass slide, dried at 46 °C for 20 min, and covered with 16 μl of low-melting-point agarose. This preparation was dried again and dehydrated in sequential dilutions of ethanol 50, 80 and 100 % for 2 min each. Hybridization was conducted using 8 μl of buffer HB (360 μl NaCl 5 M, 40.0 μl Tris-Cl pH 8.0, 1 M, 700 μl of formamide, and 4.00 μl SDS 10 % (*w*/*v*) in 2 ml final volume) and 1 μl of each labeled probe. Probes used in this work, with the corresponding fluorescent dye, were EUB338: Alexa 488 (Ex 495 nm, Em 519 nm) (Amann et al. 1995), ALF443: Cascade Blue (Ex 399 nm, Em 423 nm) (Hernández-Zárate and Olmos-Soto 2006), GAM42a: Alexa 594 (Ex 590 nm, Em 617 nm) and BET42a: Alexa 532 (Ex 532 nm, 554 nm) (Lee et al. 2011). Samples were incubated for 16 h at 46 °C in a humidity-controlled chamber and washed twice using 50 μl of buffer WS (1 ml Tris-Cl pH 8.0, 1 M, 700 μl NaCl 5 M, 500 μl EDTA 0.5 M, and 50 μl SDS 10 % (*w*/*v*) in 50 ml final volume) for 15 min at 48 °C in a

humidity-controlled chamber. Finally, slides were air-dried and prepared for microscopy with Sigma® Glycerol medium and PBS buffer. Cells were detected in a Carl Zeiss® Axiophot epifluorescence microscope, and images were obtained using 100X Plan Neo-fluor Zeiss with an exposure time of 0.5 sec. Appropriate filter sets for the specific fluorochromes were used: F2 (blue from 377 to 464 nm, green from 470 to 560 nm, and red from 592 to 675 nm), and common octocoral auto-fluorescence was evaluated by comparing with the scraped material without probes. Twenty to twenty-five pictures were taken for analysis using ImageJ software. We counted the cells detected by the EUB338 probe and calculated the difference (as percentage) with those detected by each of the selected class probes. The standard error was calculated for each comparison (Roche 2002; Zwirglmaier et al. 2003; Abràmoff et al. 2004; Lee et al. 2011).

Disc-Diffusion Antibacterial Assay The antibacterial activity of the crude *P. elisabethae* extract, fractions F8, F7, and FPST, and pure compounds PsG, PsP, PsQ, PsS, PsT, PsU, *seco*-PsJ, and *seco*-PsK, were evaluated using the standard paper disc-diffusion assay (National Committee for Clinical Laboratory Standards 2004) and following the procedure described previously by Newbold et al. (1999) and Mora-Cristancho et al. (2011). The aforementioned treatments were tested against six bacterial strains isolated from heavily fouled marine surfaces. Extract (15 $\mu g \mu l^{-1}$), fractions (15 $\mu g \mu l^{-1}$), pure compounds (1.5 $\mu g \mu l^{-1}$), and positive controls (cuprous oxide, cupric sulfate, tetracycline, and kanamycin; 1.5 $\mu g \mu l^{-1}$) were dissolved in methanol and 20 µl loaded onto paper discs (Whatman No. 1; disc diam=5.2 mm). Methanol was used as negative control. The amount of extract or fractions assayed was chosen because it represents the approximate volumetric capacity of each disc (Newbold et al. 1999).

The bacterial strains *O. pseudogringnonense* strains 1 and 2, *A. macleodii*, *O. iheyensis*, *Kocuria* sp., and *Bacillus* sp., were grown in a suitable marine broth and incubated for 24 h at 25 °C. One hundred microliters of bacterial culture, adjusted with sterile broth to the 0.5 McFarland-standard, were inoculated onto marine agar Difco 2216 plates, and each paper disc was placed on the agar in triplicate. The plates were incubated at 25 °C for 24 h. The diameter of the inhibition zone was measured after 24 h, and the measurements were confirmed after 48 h.

Additionally, the FPST fraction containing a clean mixture of Ps and *seco*-Ps was evaluated against the culturable surface associated bacteria of *P. elisabethae* using the same methodology described above. In this case, only kanamycin and tetracycline were used as positive controls.

Biofilm Inhibition Assay The bacterial biofilm assay was performed according to the procedure described by O'Toole et al. (2000) using polystyrene microtitre plates (96 wells). Pre-inoculums of *P. putida* IsoF, *O. pseudogringnonense* strains 1 and 2, *A. macleodii*, *O. iheyensis*, *Kocuria* sp., and *Bacillus* sp., were grown in LB and Marine Medium Difco 2216, respectively. Once cultures reached an OD of 0.3–0.4 at 595 nm, 100 µl per well were inoculated on polystyrene microtitre plates. Varying final concentrations (100, 50, and 10 $\mu g ml^{-1}$) of PsG, PsP, PsQ, PsS, PsT, PsU, *seco*-PsJ, and *seco*-PsK were used per well, including kanamycin (50 $\mu g ml^{-1}$) and tetracycline (15 $\mu g ml^{-1}$) as positive controls. The concentration of methanol used in order to solubilize the compounds never exceeded 0.5 % in each well. Finally, culture medium was added to 200 µl.

Microtitre plates were incubated for 24 h at 37 °C. After discarding the cultured medium by inversion, the wells were rinsed three times with distilled water (200 µl per well). The bacterial biofilm remaining on the well walls was stained for 5 min with 200 µl per well of crystal violet (1 %*w*/*v* solution of ethanol/acetone (80:20*v*/*v*)). The colorant was removed by inversion, and each well was rinsed with distilled water (200 µl per well). Differential staining was quantitated at 620 nm using a BIORAD 550 microplate reader. For each experiment, the correction for background staining was performed by subtracting the value of non-inoculated controls.

Statistical Analyses Results are presented as mean±standard error of mean (S.E.M.). Growth inhibition and Biofilm inhibition data were subjected to descriptive statistics and analysis of variance (ANOVA) and complemented by Dunnet and Bonferroni multiple comparisons. P values <0.05, <0.01 and <0.001 were considered statistically significant. Statistical analyses were performed using GraphPad Software, Prism V. 5.0.

Results

Isolation and Identification of Ps and seco-Ps from P. elisabethae From the dichloromethane:methanol (1:1) extract of *P. elisabethae*, the non-polar fractions F8 and F7, and the polar fraction FPST (a clean mixture of Ps and *seco*-Ps) were obtained. Subsequently, the major components: PsG, PsP, PsQ, PsS, PsT, PsU, *seco*-PsJ and *seco*-PsK (Fig. 1) were isolated from FPST, carefully identified by spectroscopic means (Fig. S1, Tables S1, S2), and their purities were checked by HPLC. Furthermore, the components of the FSPT fraction were quantitated by LC-MS using the total ion current trace (TIC), revealing the following composition (grams of compound per 100 g of octocoral dry mass): amphilectosin A (Amp A, 0.2 %), amphilectosin B (Amp B, 0.2 %), PsG (1.2 %), PsK (0.4 %), PsP (0.8 %),

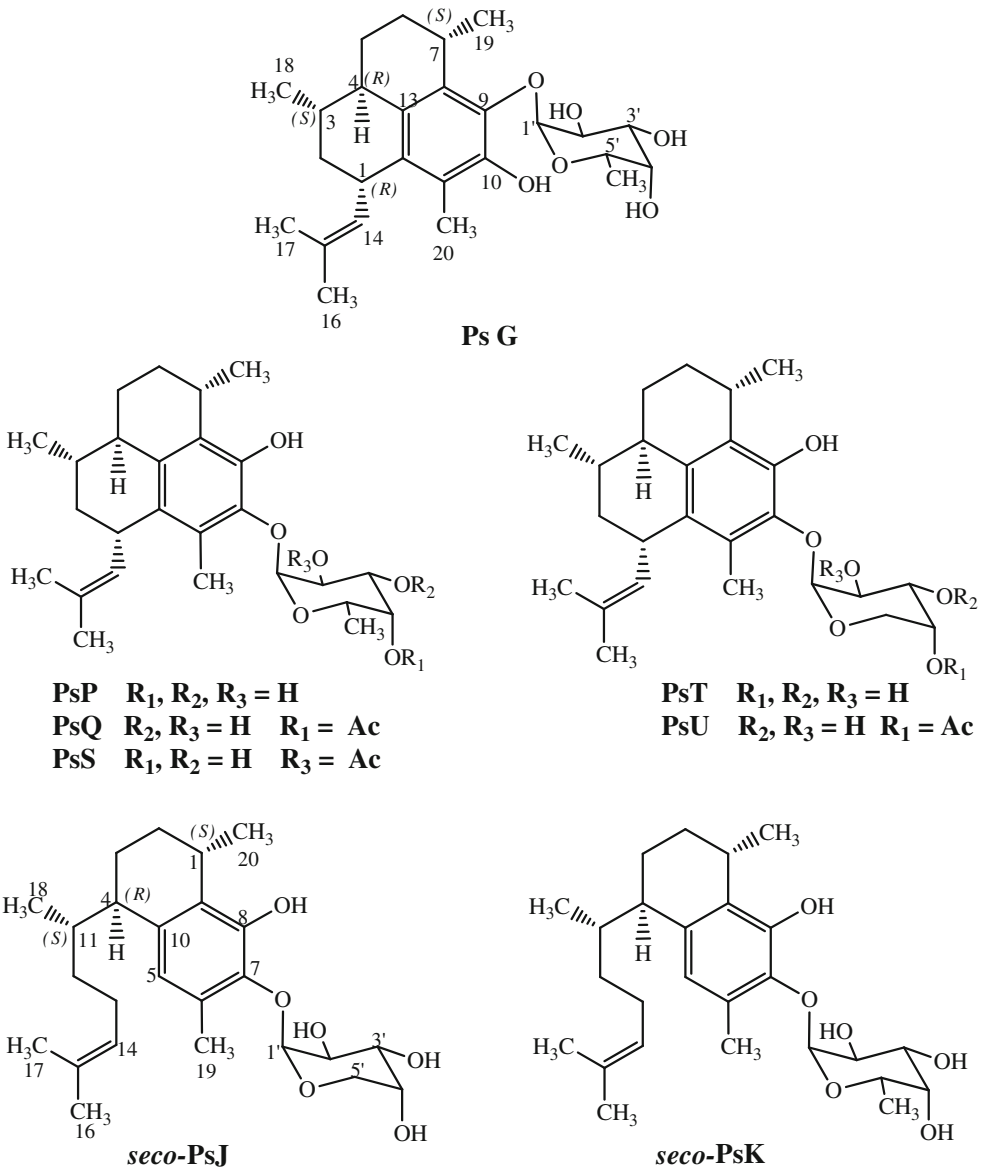

Fig. 1 Chemical structures of Ps and *seco*-Ps isolated from *Pseudopterogorgia elisabethae* in Providencia Island

PsT (0.5 %), *seco*-PsJ (0.4 %), *seco*-PsK (0.3 %), PsQ (3.4 %), PsU (3.0 %), and PsS (0.5 %).

Antibacterial Activity Assay Against Microfouling Bacteria The diameter of complete inhibition zones±SEM (N=3) of the crude extract, non-polar fractions (F7 and F8), FPST fraction, PsG, PsP, PsQ, PsS, PsT, PsU, *seco*-PsJ, *seco*-PsK, and the positive controls Cu_2O and $CuSO_4$ (common antifouling agents), and kanamycin and tetracycline (common antibiotic agents) against the six bacteria associated with heavily fouled marine surfaces, are shown in Table 1. The crude extract was moderately active (inhibition halos: 11.4–15.3 mm) against all Gram-positive bacteria (*O. iheyensis*, *Kocuria* sp., and *Bacillus* sp.), while it just weakly inhibited the growth of one Gram-negative bacterium (*O. pseudogringnonense* Strain 2; 6.4 mm). With respect to the non-polar fractions, F8 was weakly active against *A. macleodii* (6.0 mm) and *Kocuria* sp. (5.6 mm), whereas F7 showed low activity (6.4–10.3 mm) against all bacteria, except *O. pseudogringnonense* Strain 1 where it was inactive. FPST showed similar activity (inhibition zones between 11.3 and 14.4 mm) against all Gram-positive bacteria (*O. iheyensis*, *Kocuria* sp., and *Bacillus* sp.) as shown by the crude extract; however, FPST was not active against any Gram-negative bacteria. With respect to all Ps and *seco*-Ps isolated, they were moderately to highly active against all Gram-positive bacteria assayed with similar inhibition halos (9.9–20.4 mm). As with FPST, none of these compounds showed activity against the Gram-negative bacteria assayed (*O. pseudogringnonense* strains 1 and 2 and *A. macleodii*). Among the positive controls, CuO_2 was the only treatment that inhibited the growth of both Gram-positive (11.6–12.5 mm) and Gram-negative (10.9–19.5 mm) bacteria; $CuSO_4$ was active only against the Gram-negative

Table 1 Antibacterial activity of Ps and *seco*-Ps isolated from *Pseudopterogorgia elisabethae* against six bacterial strains associated with heavily fouled marine surfaces (The sponge *Aplysina lacunosa* and the empty shell of *Donax* sp.)

Treatment/Bacterium	Dose (μg)	Diameter (mm) of the zones of complete inhibition[a], mean±SME					
		Gram-negative			Gram-positive		
		Alteromonas macleodii	*Ochrobactrum pseudogringnonense* Strain 1	*Ochrobactrum pseudogringnonense* Strain 2	*Oceanobacillus iheyensis*	*Kocuria* sp.	*Bacillus* sp.
Crude extract	300	–	–	6.4±0.2	11.4±1.5	12.9±2.6	15.3±2.9
F8	300	6.0±0.7	–	–	–	5.6±0.6	–
F7	300	9.1±1.4	–	10.3±1.0	6.4±1.7	8.1±0.8	7.4±2.3
FPST	300	–	–	–	14.4±2.0	11.3±0.8	12.6±1.6
PsG	30	–	–	–	20.4±5.0	10.9±2.0	11.6±2.4
PsP	30	–	–	–	12.7±0.8	11.2±0.5	10.7±2.2
PsQ	30	–	–	–	11.7±1.6	9.9±0.3	9.3±2.4
PsS	30	–	–	–	12.6±1.5	12.2±2.7	8.9±1.6
PsT	30	–	–	–	13.5±5.9	16.2±2.7	10.8±6.0
PsU	30	–	–	–	16.5±2.9	15.2±2.7	17.8±6.0
seco-PsJ	30	–	–	–	14.5±4.3	13.3±1.6	11.3±1.9
seco-PsK	30		–	–	12.9±1.3	10.3±2.6	8.3±0.9
Tetracycline	30	–	–	–	11.6±0.2	12.9±2.1	17.3±0.6
Kanamycin	30	14.6±0.3	–	8.5±0.6	20.5±0.3	22.1±0.2	21.1±0.3
Cu_2O	30	12.5±0.8	11.7±1.5	11.6±0.8	19.5±1.0	10.9±1.2	11.8±0.2
$CuSO_4$	30	–	–	–	–	–	18.4±0.6

F8: Fraction 8; F7: Fraction 7; FPST: polar fraction containing a mixture of Ps and *seco*-Ps; PsG: Pseudopterosin G; PsP: Pseudopterosin P; PsQ: Pseudopterosin Q; PsS: Pseudopterosin S; PsT: Pseudopterosin T; PsU: Pseudopterosin U; *seco*-PsJ: *seco*-Pseudopterosin J; *seco*-PsK: *seco*-Pseudopterosin K; Cu_2O: cuprous oxide; $CuSO_4$: cupric sulfate.

[a] Includes the disc diameter (5.2 mm) $n=3$

bacterium *Bacillus* sp. (18.4 mm), tetracycline showed activity against all Gram-positive bacteria (11.6–17.3 mm), and kanamycin was active against every evaluated bacteria, both Gram-positive (8.5–12.5 mm) and Gram-negative (20.5–22.1 mm), excluding *O. pseudogringnonense* strain 1 (Gram-negative).

Biofilm Inhibition Assay The effects of Ps and *seco*-Ps (100, 50, and 10 $\mu g ml^{-1}$), kanamycin (50 $\mu g ml^{-1}$), and tetracycline (15 $\mu g ml^{-1}$) on bacterial growth and on biofilm disruption (% of inhibition) of *P. putida* IsoF as well against the six bacterial strains is shown in Fig. 2 ($N=3$). Ps and *seco*-Ps had two clearly differentiated effects against Gram-positive and Gram-negative bacteria. In the first, these compounds showed no activity (did not inhibit bacterial growth) and did not promote biofilm formation in the tested Gram-negative bacteria (*P. putida* IsoF, *A. macleodii*, *O. pseudogringonense* strains 1 and 2) (Fig. 2a). In contrast, these compounds were able to promote growth and exerted a remarkable effect on the biofilm formation of *P. putida* IsoF, *A. macleodii* and *O. pseudogringonense* strains 1 and 2. Interestingly, the effect against Gram-negative bacteria was concentration-dependent, i.e., a higher concentration of Ps or *seco*-Ps increased biofilm formation in those bacteria. The second observed effect (Fig. 2b) was that the Ps and *seco*-Ps inhibited both growth and biofilm formation of Gram-positive bacteria (*O. iheyensis*, *Kocuria* sp., and *Bacillus* sp.). However, these results do not confirm whether biofilm inhibition resulted from specific inhibition of the bacterial surface association because those compounds also affected growth of those strains. These results confirmed the data obtained in the disc diffusion antibacterial assay (Table 1).

Pseudopterogorgia elisabethae *Bacterial Composition*. For purposes of this study, the three bTEFAP 16S rDNA libraries reported by us in Correa et al. (unpublished), were averaged for comparison with the FISH abundance data. Figure 3a shows the taxonomical composition—at Phyla level—of the total bacterial community associated with *P. elisabethae*. As previously reported, the total bacterial community consisted of a range of 12 unique phyla (Chloroflexi, Lentisphaerae, Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria, Proteobacteria, Bacteroidetes, Spirochaetes, Firmicutes, Actinobacteria, and Fusobacteria). Additional sequences

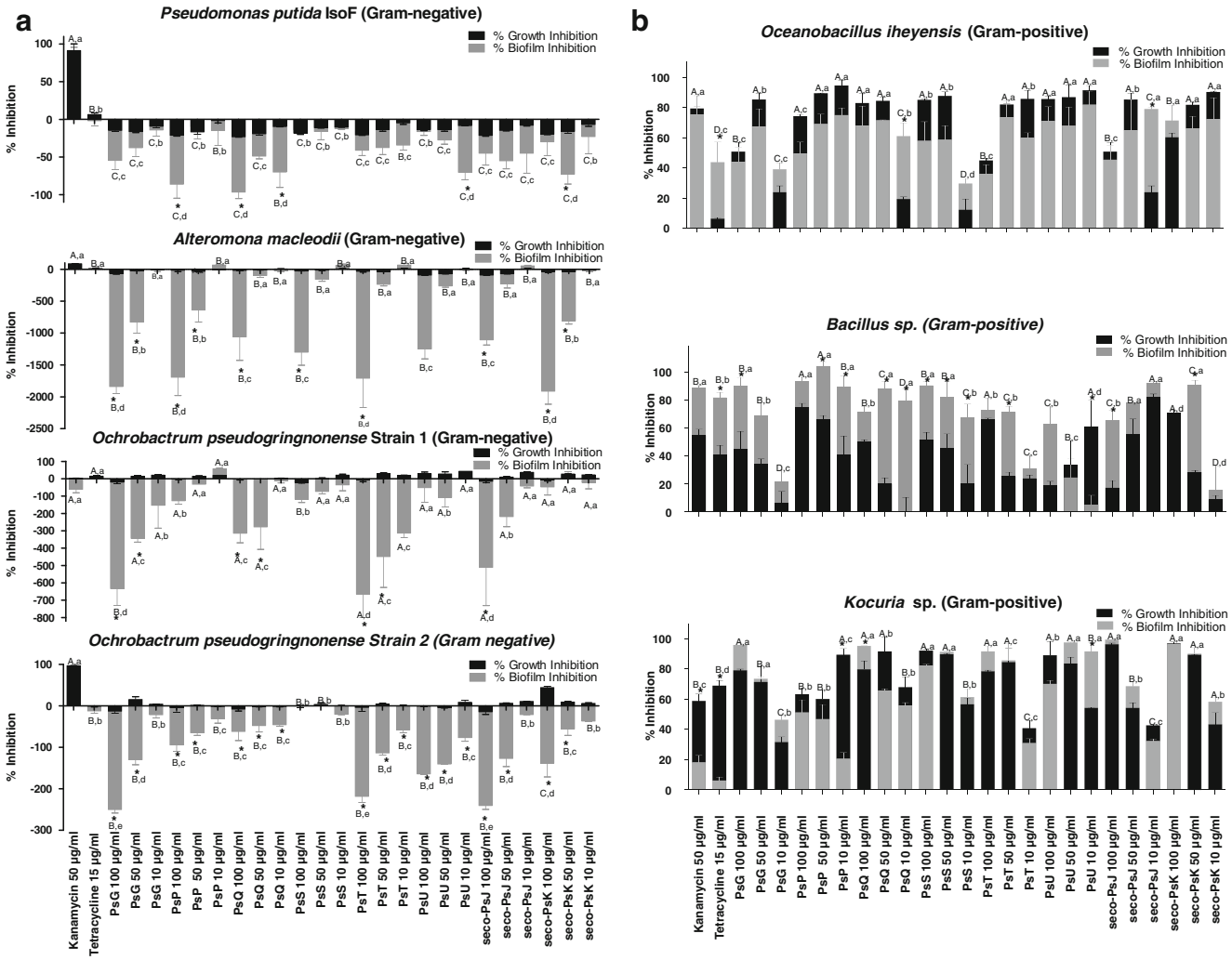

Fig. 2 Effect of the Ps and *seco*-Ps isolated from *Pseudopterogorgia elisabethae* on the growth and biofilm formation of **a** Gram-negative bacterial strains associated with heavily fouled marine surfaces and *Pseudomonas putida*; **b** Gram-positive bacterial strains associated with heavily fouled marine surfaces. Data expressed as mean±S.M.E., $n=3$ (Anova post-test Bonferroni multiple comparison: *$P<0.05$ between % Growth inhibition and % Biofilm inhibition). The same letter (*A*, *B*, *C*, *D* and *E* for %Growth Inhibition; and *a*, *b*, *c*, *d*, and *e* for %Biofilm formation) indicates no significant difference between treatments (Anova post-test Dunnet $P<0,05$, $P<0,01$ and $P<0,001$) for each bacterium

corresponding to less than 20 % of the total community, were classified as bacteria but could not be further identified (Bacteria non-classified). Proteobacteria (75.03 %) was the most abundant phylum followed by Cyanobacteria (2.96 %), Bacteroidetes (2.27 %), and Actinobacteria (0.73 %). In the Proteobacteria phylum, Gammaproteobacteria was the most abundant class (91.56 %) followed by Alphaproteobacteria (7.45 %), Deltaproteobacteria (0.15 %), and Betaproteobacteria (0.12 %). Since the most abundant phylum was Proteobacteria, we decided to use FISH probes to analyze the class composition of surface-associated bacteria on *P. elisabethae*. Using specific probes, we detected that 92.0 % of bacterial cells belonged to the Proteobacteria phylum, in agreement with our previous findings (Correa et al. unpublished). The proportions also were consistent between those two culture-independent techniques at the class level. FISH analysis showed that most abundant surface-associated cells belonged to the Gammaproteobacteria (45.6 %) class, followed by Alphaproteobacteria (37.0 %), and Betaproteobacteria (17.4 %). Figure 3b shows the average percentage of cells detected by each specific probe EUB338, ALF443, GAM42a, and BET42a. Epifluorescence micrographs also show the higher proportion of Gammaproteobacteria (red-yellow) compared to Alphaproteobacteria (blue-green) and Betaproteobacteria (yellow) (Fig. 4). In Fig. 4a and b, Eubacteria (green) could be observed together with Alphaproteobacteria (blue-green) and Gammaproteobacteria (yellow), respectively. For Betaproteobacteria, the comparison was made independently because the few bacterial cells detected with the BET42a probe could not be accurately distinguished with the filter settings used. Figure 4c shows

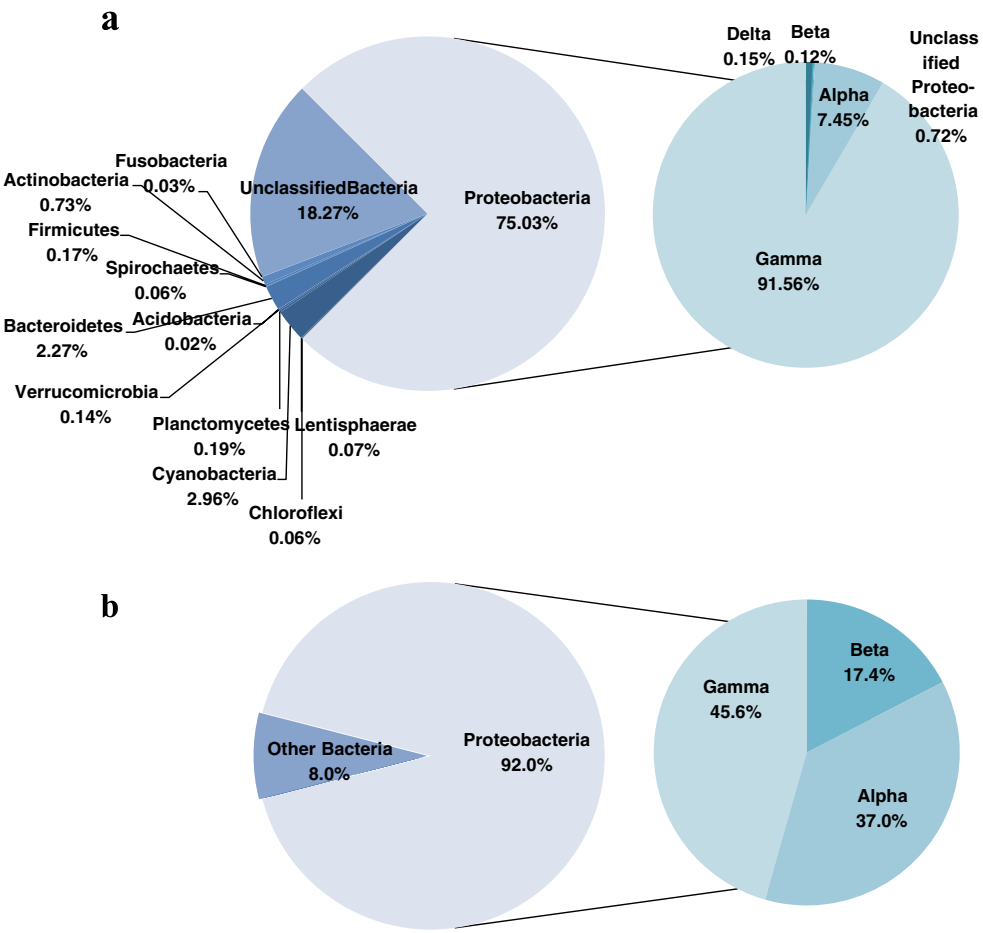

Fig. 3 Taxonomic composition of the bacterial community associated with *Pseudopterogorgia elisabethae*. **a** Composition estimated by bTEFAP 16S rDNA Libraries (*N*=3), those date were taken from Correa et al. 2012. General classification, "Unclassified_bacteria": the sequences which were not classified into any known Phylum and "Unclassified_Proteobacteria": the sequences which were not classified into any know Proteobacteria Class. **b** Average percentage of bacterial cells detected by FISH using each specific probe. Bacteria: EUB338, Alphaproteobacteria: ALF443, Gammaproteobacteria: GAM42a and Betaproteobacteria: BET4a

the Betaproteobacteria cells detected with the BET42a probe and Fig. 4d shows bacterial cells hybridized with only the EUB338 probe in order to detect Eubacterial cells.

Antibacterial Activity Against Culturable Surface Bacteria of P. elisabethae The FPST fraction (containing a clean mixture of Ps and *seco*-Ps) was used to evaluate the effect of the Ps and *seco*-Ps against the 26 bacteria previously isolated from the octocoral surface (Correa et al. unpublished). Table 2 shows the diameter of complete inhibition zones with the FPST (300 μg) and the antibiotics used as controls (kanamycin and tetracycline, 30 μg each). FPST exhibited a selective bactericidal effect against Gram-positive culturable bacteria belonging to the phyla Actinobacteria (inhibition halos 9.3–23.3 mm) and Firmicutes (6.7–13.0 mm), whereas it did not show activity against any Gram-negative bacteria belonging to the Proteobacteria phylum. For the antibiotic agents, both kanamycin (6.7–28.3 mm) and tetracycline (8.7–18.3 mm) were active against the majority of the bacteria evaluated. *Staphylococcus warneri, Pseudomonas asplenii*, and *Microbacterium luteolum* were not sensitive to any antibacterial controls. *Oceanobacillus profundis, Tetrathiobacter kashimirensis*, and *Brachybacterium conglomeratum* were not sensitive to kanamycin and *Micrococcus luteus* was not sensitive to tetracycline.

Discussion

Marine sessile organisms have developed several mechanisms to face competitive situations such as predation, pathogenicity, and fouling. In many reef organisms, the importance of chemical defenses against predation and other interspecific interactions has been assessed (Pawlik 1993, 2011; Hay 1996; O'Neal and Pawlik 2002; Changyun et al. 2008; Paul and Ritson-Williams 2008). Gorgonians in particular have been recognized as being chemically defended against predation (O'Neal and Pawlik 2002; Changyun et al. 2008), and microbial infestation (Kim 1994; Kim et al. 2000a, b; Koh et al. 2002). Conversely, less is known about the ability of gorgonians to remain mostly free of macrofouling.

Fouling is a multi-step process that culminates in the establishment and development of a community composed of prokaryotes, fungi, protists, and invertebrates. Bacterial biofilms have long-been recognized as fundamental settlement

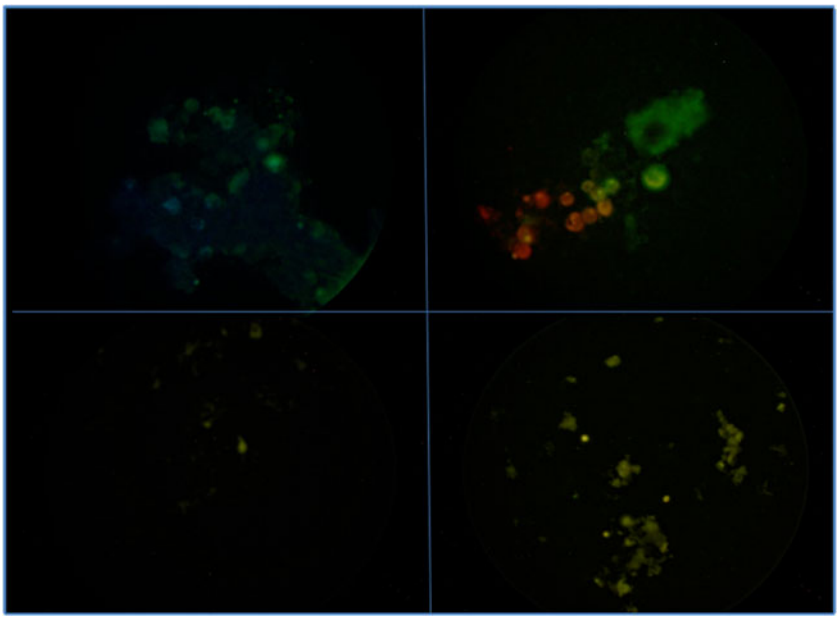

Fig. 4 Epifluorescence micrographs of bacteria found in the scraped material from the surface of fragments of *Pseudopterogorgia elisabethae*. Cells were visualized using specific FISH probes for Eubacteria (EUB338—*green*), Alphaproteobacteria (ALF443—*blue*), Gammaproteobacteria (GAM42a—*red*) and Betaproteobacteria (BET42a—*yellow*). Panel **a**. Cells hybridized with probes EUB338 and ALF443. Panel **b**. Cells hybridized with probes EUB338 and GAM42a. Panel **c**. Cells hybridized with probe BET42a. Panel **d**. Cells hybridized with probe EU338. Scales bars: 5 μm

cues for many invertebrate larvae that colonize hard substrata, such as sponges, cnidarians, mollusks, barnacles, bryozoans, and ascidians (Fusetani 2011). This intrinsically complex process is the result of a network of interactions in the pioneering biofilm and subsequently in the community of colonizers (Joint et al. 2002). It is important to mention that no laboratory bioassay could replicate such a complex process because it involves a wide range of physical, chemical, and biological interactions (Briand 2009). Recently, some technical approximations have been reported as indicators of the antifouling potential of chemical compounds, using as a model the growth inhibition bioassay and the disruption of biofilm formation in bacteria associated with heavily fouled marine surfaces (Fusetani 2011). In order to assess the potential antifouling activity of Ps and *seco*-Ps isolated from *P. elisabethae*, we tested those compounds against six bacterial strains isolated from fouled substrates to determine their sensitivity (growth inhibition) and disruption of biofilm formation. As mentioned, we wanted to determine if Ps and *seco*-Ps from *P. elisabethae* have an influence on the selection, settlement, and colonization control of bacteria on the gorgonian surface.

The crude extract of *P. elisabethae* showed antifouling activity, selectively inhibiting the growth of Gram-positive bacteria (*O. iheyensis, Kocuria* sp., *and Bacillus* sp.). On the other hand, the extract only had weak activity against a Gram-negative bacterium (*O. pseudogringnonense* Strain 2). The evaluation of the activity of the non-polar (F8 and F7) and FPST (mixture of Ps and seco-Ps) fractions, allowed us to establish that the Ps and *seco*-Ps are the compounds responsible for the antibacterial activity shown by the crude extract. As with the crude extract, the FPST fraction showed considerable selective activity against Gram-positive bacteria. The non-polar fractions showed weak and non-selective activity against some of the bacteria assayed. In the same experiment, all pure Ps and *seco*-Ps showed effects similar to those of FPST.

Chemical defenses may affect microorganisms by directly inhibiting their growth, by inducing a negative chemotactic response or by attracting favorable epibionts that competitively exclude deleterious microorganisms (Jensen et al. 1996). Other authors have suggested that the release or exudation of chemical compounds may preclude the establishment of microbial biofilms and thus fouling (McCaffrey and Endean 1985; Becerro et al. 1994; Steinberg et al. 2001; Harder et al. 2012). The microscale distribution of chemical cues or settlement deterrents *in situ* has not been successfully determined due to difficulties in the collection, characterization, and quantification of those cues, as well as in determining the role that hydrodynamic factors play on a community scale (Steinberg et al. 2001). For a compound to play a role in preventing fouling, it should be surface-associated or continuously released to deter fouling organisms (Steinberg et al. 2001). To date, there is no evidence that either pseudopterosins or other compounds from P. *elisabethae* are released into the surrounding sea water or that they are surface-associated. That is an assumption based on the fact that the surface of this gorgonian is always very "clean". In some red algae, soft corals, and sponges, it has been demonstrated, by direct or indirect evidence, that antimicrobial, larvicidal, or allelopathic compounds are released into the surrounding seawater with a clear advantage to the producing organism (reviewed in Steinberg et al. 2001). The concentrations tested in the disk diffusion assay were likely overestimations of natural concentrations and concentrations faced by microorganisms *in situ* (Newbold et al. 1999; Kelly et al. 2005) However, the production of

Table 2 Antibacterial activity of FPST (Ps AND *seco*-Ps) isolated from *Pseudopterogorgia elisabethae* against culturable surface bacteria associated with *P. elisabethae*

Code	Bacteria (Accession number GenBank|)[a]	Diameter (mm) of the zones of complete inhibition[b], mean±SME		
		FPST (300 μg)	Kanamycin (30 μg)	Tetracycline (30 μg)
Firmicutes (Gram-positive)				
RKHC-48	*Staphylococcus auricularis*(JQ282819.1)	6.7±0.9	15.0±1.6	11.0±0.8
RKHC-78A	*Staphylococcus epidermis* (JQ282825.1)	11.0±2.0	9.0±0.5	10.3±0.9
RKHC-78B	*Staphylococcus warneri* (JQ282826.1)	8.7±0.7	–	–
RKHC-9	*Bacillus cereus* (JQ282814.1)	20.3±0.7	9.0±1.4	8.7±1.1
RKHC-21	*Bacillus licheniformis* (JQ282816.1)	14.3±2.7	11.3±2.0	20.0±1.3
RKHC-37	*Bacillus mojavensis* (JQ282818.1)	10.0±0.7	20.0±2.4	14.3±2.3
RKHC-68A	*Bacillus horneckiae* (JQ282823.1)	10.0±1.5	19.0±1.8	8.0±1.7
RKHC-69A	*Bacillus anthracis* (JQ282824.1)	13.0±2.7	17.3±0.8	9.0±0.6
RKHC-62B	*Oceanobacillus profundis* (JQ282822.1)	11.7±0.9	12.7±2.1	–
RKHC-8	*Paenibacillius glucanolyticus* (JQ282827.1)	11.3±2.0	15.7±1.5	12.0±0.6
Proteobacteria (Gram-negative)				
RKHC-25	*Pseudomonas oleovorans* (JQ282833.1)	–	12.5±0.9	16.3±2.4
RKHC-61A	*Pseudomonas grimonti* (JQ282836.1)	–	28.3±1.2	9.7±1.1
RKHC-42	*Pseudomonas moraviensis* (JQ282834.1)	–	–	–
RKHC-67B	*Vibrio sinaloensis* (JQ282838.1)	–	14.5±0.3	14.5±1.7
RKHC-76A	*Vibrio maritimus* (JQ282839.1)	–	10.0±1.1	11.7±1.9
RKHC-23	*Photobacterium eurosenbergii* (JQ282832.1)	–	9.0±1.0	11.7±1.4
RKHC-60A	*Tetrathiobacter mimigardefordensis* (JQ282835.1)	–	16.0±1.3	–
RKHC-14	*Ruegeria atlantica* (JQ282831.1)	–	11.7±1.8	13.0±1.3
Actinobacteria (Gram-positive)				
RKHC-26	*Rhodococcus baikonurensis*(JQ282804.1)	13.0±0.7	9.0±0.9	9.0±0.5
RKHC-35	*Corynebacterium afermentas* (JQ282807.1)	11.0±2.3	10.3±1.7	18.3±2.7
RKHC-45	*Microbacterium luteolum* (JQ282808.1)	23.3±3.0	–	–
RKHC-11	*Microbacterium phyllosphaerae* (JQ282803.1)	19.7±3.0	11.7±0.7	8.0±2.0
RKHC-49	*Kocuria kristinae* (JQ282809.1)	9.3±1.7	6.7±0.3	15.3±0.7
RKHC-66A	*Brevibacterium casei* (JQ282811.1)	16.7±2.3	9.7±0.6	12.7±1.3
RKHC-33	*Brachybacterium conglomeratum* (JQ282806.1)	21.7±2.7	9.3±0.7	–
RKHC-71A	*Micrococcus luteus* (JQ282813.1)	19.0±3.8	–	17.0±1.4

[a] Characterization was done by sequencing of the 16S rRNA gene (Correa et al. 2012

[b] Includes the disc diameter (5.2 mm) $n=3$

antibacterial metabolites is probably not the only method to control bacterial colonization and surface fouling (Kelly et al. 2005) and microbe-microbe interactions may be important as well.

It has been reported for some species belonging to the genus *Pseudopterogorgia* that the maximum concentrations of terpenes occur in polyps (Harvell and Fenical 1989). This also may be the case in *P. elisabethae*, where the polyps could have high concentrations of Ps and *seco*-Ps to prevent the settlement of surface colonizing bacteria thus avoiding the following fouling stages. This is supported by the fact that colonies of *P. elisabethae* subjected to axial tissue grazing by the gastropod *Cyphoma gibbosum* and the polychaetes *Hermodice* spp., may have their polyps damaged, favoring colonization by algae, sponges, tunicates, and other invertebrates on the exposed axial skeleton (Castanaro and Lasker 2003).

Additionally, Ps and *seco*-Ps were able to induce biofilm formation of Gram-negative marine bacteria in polystyrene microtitre plates. This fact leads us to suggest that *P. elisabethae* may produce compounds that bring to the holobiont (holobiont = octocoral + microbiota) a chemical selection mechanism for prokaryotic colonizers, in this case Gram-negative bacteria, thus avoiding by competition the colonization of Gram-positive bacteria. By means of this mechanism, the gorgonian could prevent the settlement of potential foulers or fastidious bacteria. These findings reflect the importance of chemical cues in the establishment of

the native microbiota and the sequential development of associated and symbiotic communities in marine invertebrates. This study suggests that Ps and *seco*-Ps may act as specific inhibitors of microfouling communities, eventually preventing the establishment of later successional stages of macrofoulers (macroalgae and invertebrates). Therefore, we stress the significance of chemical substances in the holobiont, that may offer an advantage for gaining protection and establishing equilibrium for the entire organism.

As mentioned, Ps and *seco*-Ps appear to modulate bacterial growth (inhibiting the growth of certain Gram-positive bacteria and inducing the association of Gram-negative bacteria to surfaces), which suggests an ecological role of these compounds in the chemical modulation of epibiosis on the surface of *P. elisabethae*. The indirect selection of certain bacteria on the surface may control special signaling for larval settlement (Huang and Hadfield 2003); thus, these compounds might be involved in the control of microbiota composition on the surface of *P. elisabethae*, deterring fouling larvae or algal spores. The active selection of the microbiota composition on *P. elisabethae* could be the mechanism by which *P. elisabethae* keeps an unfouled surface. Similar mechanisms have been reported in the alcyonacean *Dendronephtya* (Harder et al. 2003).

We also studied whether the selective antibacterial activity of Ps and *seco*-Ps favors the establishment of Gram-negative bacteria on the surface of *P. elisabethae* by characterizing the total bacterial community associated with this gorgonian by using two different approaches: FISH and bTEFAP. Our results show that Ps and *seco*-Ps production could be an important factor in controlling the growth of surface-associated bacteria and actively encouraging the growth of Gram-negative bacteria, since those were invariably the most abundant bacteria on the surface of this gorgonian.

Pyrosequencing results have shown that the natural abundance of Gram-positive bacteria phyla (Planctomycetes, Verrucomicrobia, Firmicutes and Actinobacteria) associated with *P. elisabethae* is quite low (1.23 %), whereas bacteria belonging to Gram-negative phyla (Chloroflexi, Lentisphaerae, Cyanobacteria, Acidobacteria, Proteobacteria, Bacteroidetes, Spirochaetes, and Fusobacteria) account for 80.50 % of the total community, being Gammaproteobacteria the most abundant class (91.56 %) (Correa et al. unpublished). The same pattern was observed when using FISH for surface-associated bacteria, three Proteobacteria classes represented 92.0 % of the detected cells: Gammaproteobacteria (45.6 %), Alphaproteobacteria (37.0 %) and Betaproteobacteria (17.4 %). These results indicate the predominance of Gram-negative cells on the surface of *P. elisabethae*. In addition, the antibacterial assay against *P. elisabethae* cultivable symbiont bacteria suggests that Ps and *seco*-Ps production could be a factor in controlling the growth of surface-associated bacteria. Moreover, the biofilm assay shows that these compounds actively encourage the growth of Gram-negative bacteria. Together, this suggests that Ps and *seco*-Ps do exert a chemical influence on the settlement of these bacteria. Whether those compounds are exuded or surface-associated in the gorgonian is a matter of debate as in many other marine invertebrates, since experimental approaches to prove this remain complex.

Harder et al. (2003) evaluated the chemical mechanism against bacterial epibiosis in the soft coral *Dendronephthya* sp. They evaluated organic extracts from the coral against coral-associated bacteria (from coral swabs) and also against 33 isolates of benthic bacteria that had settled on clean glass slides deployed in the field. They found that coral associated bacteria (γ-Proteobacteria, α-Proteobacteria, and Cytophaga-Flexibacter-Bacteroides) were not affected by coral extracts, whereas the growth and attachment of benthic bacteria was largely suppressed by coral extracts, especially those of medium polarity. The authors went a step further and evaluated the inhibition of waterborne products of coral associated bacteria on benthic bacteria, finding that these products could also inhibit some of the benthic bacteria by the same mechanisms detected in the coral extracts. The authors suggested wider resistance mechanisms or the establishment of a symbiotic interaction of the coral and its associated bacteria that might provide an exogenous defense mechanism against epibiosis by deleterious bacteria.

To verify that Ps and *seco*-Ps actually do have an effect on the bacterial community, we evaluated a mixture of these compounds (FPST) on surface-associated culturable bacteria isolated from *P. elisabethae*. We used 26 bacterial strains originally isolated from the surface of *P. elisabethae*. Our results showed that FPST were active against Gram-positive bacteria (Firmicutes and Actinobacteria) but inactive against all tested Gram-negative bacteria (Proteobacteria) (Table 2), supporting our fundamental hypothesis of the active chemical selection of Gram-negative bacteria on the surface of this gorgonian species.

In recent years, the complex associations and the relation among microorganisms, marine invertebrates, and the molecules secreted by either of the organisms in the association have received greater attention due to their influence on the functional behavior of the organisms, protection against predation, or settlement and colonization on the invertebrates' surfaces (Mouchka et al. 2010). In the case of *P. elisabethae*, associated dinoflagellate symbionts (*Symbiodinium* sp., Dinophyta) are involved in the biosynthesis of PsA-D in specimens from the Bahamas (Mydlarz et al. 2003; Newberger et al. 2006). As dinoflagellates themselves are host to a plethora of microorganisms (Ashton et al. 2003), the source of these natural products remains unclear. Nonetheless, it is clear that a better understanding of the organisms responsible for the Ps and *seco*-Ps biosynthetic pathway is required to address questions

such as the ecological significance of these compounds and their beneficial role in the holobiont.

In summary, the experiments performed in this study suggest that Ps and *seco*-Ps play a role in the selection of the organisms associated with the gorgonian surface and in the regulation of associated organisms, specifically in the regulation of the resident surface bacterial community, thus leading to an unfouled surface in *P. elisabethae*. Additional studies are required to characterize the role of this bacterial selection in the defense mechanism of the *P. elisabethae* holobiont.

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